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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL PATENT APPLICATION under 37 CFR §1.53(c).

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TITLE OF THE INVENTION (500 characters max)

Anti-infectious, Prophylactic, and Therapeutic Agents and Methods to Determine Susceptibility of Individuals to Pathogens

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[X] No

[ ] Yes, the name of the U. S. Government agency and the Government contract number are: ____

Respectfully submitted,

SIGNATURE ____ Date **December 5, 2003**

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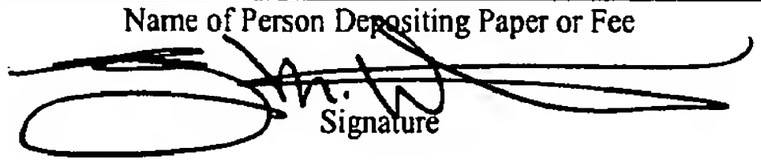
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PROVISIONAL PATENT APPLICATION
Under 37 C.F.R. § 1.53(c)

Anti-infectious, Prophylactic, and Therapeutic Agents and Methods to Determine Susceptibility of Individuals to Pathogens

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A. SPECIFIC AIMS

The primary hypothesis of this proposal is that an effective inhibitor of campylobacter and cholera binding can be synthesized based on 2′-fucosyllactose (2′-FL), a major human milk oligosaccharide, and/or its homolog or glycoconjugate; and that when given in doses equivalent to or greater than that found in human milk it will be tolerable and effective in preventing campylobacter and cholera infection in animal models. We further hypothesize that specific 2′-FL inhibitors can be more efficiently synthesized through state-of-the-art molecular biological genetic engineering techniques. The specific aims of this supplemental grant proposal will accelerate and extend those of the parent grant, and are to:

1. Synthesize polyvalent forms of 2′-FL, a human milk trisaccharide, and determine if inhibition of binding by campylobacter and by cholera *in vitro* are enhanced.
2. Test the most promising anti-campylobacter and anti-cholera oligosaccharide structure(s) in animal models.
3. Investigate novel routes for the efficient synthesis of large quantities of the protective compound.

The success of this supplementary proposal should translate our ongoing basic research into a material that is ready for testing in clinical trials.

B. BACKGROUND AND SIGNIFICANCE

Consumption of human milk is one of the most cost-effective strategies known to medicine for protecting infants against morbidity and mortality due to infectious disease (7-15). Human milk may be considered a natural and efficacious "nutriceutical," i.e., a model food that conveys immunologic benefits. Protection against infectious diseases occurs through a variety of complementary mechanisms found in human milk, including oligosaccharides and their related glycoconjugates (7,13,14,16-19). Significantly enhanced immunologic protection by breastfeeding has been demonstrated for diarrheal diseases, respiratory tract illnesses, bacteremia and meningitis, and necrotizing enterocolitis (8,20). Protection by breastfeeding is especially efficacious against diarrheal disease (8-12).

Oligosaccharides and their related glycoconjugates are major components of the innate defense system found in human milk (7,21-24). These substances, which vary from 3 to 32 sugars in size, constitute the third-most common solid component of human milk after lactose and lipid, but their role is immunologic rather than nutritive. Oligosaccharides appear to have several different immunologic functions. Several types of oligosaccharides, including the fucosyloligosaccharides and some of the sialylated oligosaccharides in human milk, have been shown to have prebiotic properties, i.e., selective stimulation of the growth of beneficial bacteria in the intestine (25,26). Our research, however, suggests an even more important role for oligosaccharides as pathogen-binding inhibitors. Protection against specific pathogens has been described for both fucosylated and sialylated human milk oligosaccharides (5,6,24,27,28). Both the fucosylated oligosaccharides and the sialylated oligosaccharides may have structural homology to cell receptors for enteropathogens, and thereby inhibit pathogen binding (24,29,30). Certain pathogens are thought to bind to sialic acid containing receptors, including EPEC, rotavirus, *Haemophilus influenzae* and other pathogens (30-33). In addition to the unbound oligosaccharides, protection by glycoconjugated substances in human milk has been demonstrated by *in vitro* studies and/or animal models against labile toxin and cholera toxin, heat-stable enterotoxin of *E. coli*, campylobacter, shiga toxin, *Streptococcus pneumoniae* and rotavirus (16,17,24,34). Lactadherin, a 46-kDa glycoprotein, has been found to vary in concentration in human milk, and we have reported significant protection against symptomatic rotavirus infection associated with increasing concentrations of lactadherin in maternal milk (34).

The focus of our renewed program project grant (2003-08) and of this supplemental application is on the human milk oligosaccharides and their conjugates that contain an α1,2-linked fucose. These oligosaccharides have been shown to inhibit host cell binding to several enteric pathogens that are the focus of our research: *Campylobacter jejuni* and cholera, stable toxin (ST)-associated *E. coli*, and major

strains of human caliciviruses (5,6,27,28). The fucose terminus of oligosaccharide structures may be connected by an α1,2 linkage catalyzed by a fucosyltransferase produced by the secretor gene (*FUT2*), or by an α1,3 or α1,4 linkage catalyzed by fucosyltransferases produced by the Lewis gene (*FUT3*) family. Polymorphisms of the secretor and Lewis genes are known to determine expression of the Lewis blood group type, fucosylated oligosaccharide patterns in human milk, and histo-blood group antigens on human epithelial cell surfaces (21,22,35). Some individuals are non-secretors (i.e, homozygous recessive for the secretor gene) who do not synthesize α1,2-linked fucosyloligosaccharides in their secretions. In Indo-European populations, the prevalence of non-secretors is approximately 20%, while in some other populations, such as Mexicans of indigenous ancestry, non-secretors are much less common (36-38). Some have shown that this heterogeneity of expression is associated with differential risk of infectious diseases in individuals and populations (5,6,28,37,39-44). At the same time, variation in concentration of protective oligosaccharides in human milk may result in breastfed infants with differing levels of protection against specific infectious diseases (21,22,36,39,45).

The most common oligosaccharides of human milk include four α1,2-linked fucosylated oligosaccharides (lacto-*N*-fucopentaose I [LNF-I], 2-fucosyllactose [2'-FL], lacto-*N*-difucohexaose I [LDFH-I] and lactodifucotetraose [LDFT]); two fucosylated oligosaccharides that are not 2-linked (lacto-*N*-fuco-pentaose II [LNF-II] and 3-fucosyllactose [3-FL]); and their two precursors (lacto-*N*-tetraose [LNT] and lacto-*N*-neotetraose [LNneoT]). These eight oligosaccharides are homologs of Lewis histo-blood group antigens, respectively: H-1, H-2, Le^b, Le^y, Le^a, Le^x, type 1 precursor, and type 2 precursor. The most commonly occurring specific α1,2-linked fucosylated oligosaccharide in human milk is 2'-FL (H-2 epitope). Comparing the composition of milks from many different mammalian species, 2'-FL is also the most conserved oligosaccharide structure, suggesting its importance in evolutionary biology (46). 2'-FL is absent, however, from the milk of some species, including cow's milk.

The relevance of human milk α1,2-linked fucosyloligosaccharides, specifically 2'-FL, as the basis of potential prophylactic and/or therapeutic agents, is described below in relation to campylobacter, cholera, and other enteric pathogens.

Relevance to campylobacter. Campylobacter strains are among the most common human and veterinary pathogens worldwide (47-54). Although diarrhea is the most frequent clinical presentation associated with campylobacter, a broad clinical spectrum is observed with this infection, including bacteremia, localized infection, and Guillain-Barre Syndrome, a severe immunoreactive complication (47,48). In the United States, the estimated incidence of campylobacter is two million symptomatic infections per year, approximately 1% of the U.S. population (49). Population-based studies in England, the U.S., and Sweden have shown a bimodal distribution, with a peak of illness in children less than 5 years of age and a second peak in adolescents and young adults 15 to 29 years old (50-54). The highest isolation rate (15 cases per 100,000) occurs in the first year of life (51). In endemic areas of developing countries, the isolation rate among children with diarrhea is 8% to 45%, with a similar rate of isolation among asymptomatic children (55,56). The annual incidence of campylobacter infections can be as high as 2.1 episodes per child-year. Foodborne infections are an emerging concern affecting millions of individuals every year. Campylobacter is the second most common cause of foodborne infection after calicivirus (49,50). The alarming increase in multiply antibiotic resistant strains of campylobacter being isolated probably results from the use of quinolones in veterinary medicine and as animal food supplements (57).

Children living in endemic areas develop effective natural immunity to campylobacter infection as the result of an intense early exposure to the organism (55,56). While immunoglobulins in human milk provide important protection against campylobacter as well as other causes of respiratory and gastrointestinal tract infections (58-60), non-immunoglobulin components in human milk also appear to play a dominant role (61-63). Among the non-immunoglobulin protective factors in human milk, the oligosaccharides and glycoconjugates appear to be the most important (30,35,45). Our data indicate that the initial steps of attachment of campylobacter to the host cell surface, critical to infection, involve binding to epithelial cell surface glycoconjugates (2,28,64). Human milk oligosaccharides with structural homology to these ligands may inhibit binding by the pathogen (2). Thus,

variable expression of these oligosaccharides in milk due to maternal genetic heterogeneity may influence the risk of infection in breastfed infants (42,65,66).

Recent advances in understanding the pathogenesis of campylobacter infection have followed the sequencing of its complete genome (67). The ability of campylobacter to adhere to and invade the epithelial cells of the ileum and cecum is well known (68-73). Motility and chemotaxis play a major role in the localization of bacteria in the lower part of the intestine (74-78). Studies of the chemotactic behavior of campylobacter have shown a positive response to the presence of fucose, but not other sugars, as well amino acids such as aspartate, cysteine, glutamate and serine (76). L-Fucose is an important constituent of both bile and mucin. These may be important factors for the affinity of the organism for the gall bladder and the lower intestinal tract. Environmental and chemotactic stimuli specifically upregulate the *C. jejuni flaA* sigma 28 promoter (77). High pH, osmolarity, and bile salts, including deoxycholate, also upregulate the *fla* promoter while high viscosity results in downregulation of the *fla* promoter. Considering that bile and mucin are mixed together in the intestine, and that *C. jejuni* colonization of the mucin layer is a prerequisite for pathogenesis *in vivo*, the overall response would be an increase in *flaA* synthesis and chemotaxis towards the mucin layer. These data explain the importance of fucose in the pathogenesis of campylobacter infection in the gastrointestinal tract (79).

Early studies demonstrated inhibition of campylobacter adherence to intestinal epithelial cells by L-fucose (80). We had shown inhibition of cell adherence *in vitro* and colonization of gut mucosa *in vivo* by fucosylated human milk oligosaccharides (28). Characterization of these human milk carbohydrate residues showed that α1,2-fucosylated oligosaccharides are the main active components, and that these oligosaccharides, particularly those containing H-2 epitopes, can inhibit campylobacter adherence to its host receptor. The specificity of binding to α1,2-fucosyl moieties was confirmed by transfecting Chinese hamster ovary (CHO) cells with the human gene for human α1,2-fucosyltransferase whose expression product catalyzes the final step of H antigen synthesis (81). While parental non-transfected CHO cells (which do not xpress H antigen) are not infected with invasive campylobacter, transfected cells are susceptible to adherence and invasion by campylobacter. The differential expression of blood group antigen H-2 at different sites of the gastrointestinal tract could explain the essential features of the pathology of campylobacter diarrhea, and likewise, the localization of infection. Mice transfected with the *FUT1* gene, flanked by the murine whey acidic protein promoter, specifically express *FUT1* in milk during lactation (82). These transfected mice produce large amounts of H-2 antigens in milk, whereas the wild type mice produce none. Pups nursing these transfected dams were protected against intestinal colonization by campylobacter. These data support the concept that H antigens are the intestinal ligands essential for the binding of campylobacter to the intestinal tract. In milk, soluble ligands containing H-2 epitopes can serve as receptor analogs that protect infants from campylobacter infection, and they may represent an important component of the innate immune system of human milk (63).

Relevance to cholera. Susceptibility to cholera appears to be related to the ABH(O) tissue-blood group antigens (83,84). Studies of immunity to experimental cholera in human volunteers showed that blood group O was significantly more frequent in volunteers who developed severe cholera (stool volume >5.0 L). A large epidemiological study done in Bangladesh demonstrated that patients with cholera were twice as likely to have O blood group as community controls (44). This study also showed that individuals with the most severe type of diarrhea were most likely to be of blood group O (68% versus 31%; $P<0.01$). One possible explanation for the increased severity of cholera in persons of blood group O is that an enhanced adherence of vibrios to the intestinal mucosa may occur in such individuals. Because of this increased susceptibility of individuals of O blood group to develop severe cholera, the immunogenicity and protective efficacy of a cholera vaccine in persons of this group have also been examined. Randomized, double-blind, placebo controlled studies with an attenuated cholera vaccine showed a stronger immune response in persons of the O blood group type, with significantly higher reciprocal geometric mean titers than the non-O roup vaccinees (85); this information supports the concept that *Vibrio cholera*e adheres more avidly to ntestinal mucosa of persons of blood group O, inducing a heightened vibrocidal response. However, the biological and molecular basis for this genetically related protection has not yet been elucidated. Previous studies have demonstrated that hemagglutination produced by *V. cholerae* with human O erythrocytes can

be inhibited with L-fucose (86). The human intestinal epithelium is rich in glycolipids and glycoproteins of the ABH(O) and Lewis histo-blood group antigens (87). The H(O) antigen consists of a backbone of fucose ·1,2DGalβ1,4... It is therefore conceivable that the H antigen serves as a receptor for *V. cholerae*. As we have previously shown in campylobacter, *V. cholerae* also binds in vitro to ABH-Lewis neoglycoproteins and also attaches preferentially to α1,2 fucose determinants expressed on the surface of FUT1-transfected CHO cells (69). There is also recent evidence that the B subunit of cholera toxin and the labile toxin (LT) of enterotoxigenic Escherichia coli not only binds with high affinity to GM1 ganglioside, but LTB also interacts with N-acetyl lactosamine-terminated glycoconjugates (87,88). It will be important to define whether 2'-FL or 2'-FLNAc inhibit colonization and intestinal fluid accumulation in experimental *V. cholerae* infection; this question is being addressed in the first (current) year of our program project grant.

Relevance to other pathogens. A growing body of research suggests that common mechanisms of pathogenesis may exist between some bacterial and viral pathogens, including the association between histo-blood group type and susceptibility to bacterial and viral diseases (2,3,5,6). An association between Lewis and secretor histo-blood group genotypes appears to be associated with a number of different pathogens. For example, Ikehara and others have reported that Lewis and secretor histo-blood group genotypes are associated with differing risk of infection with *Helicobacter pylori* (42). Huang et al and have reported that secretor blood group individuals have increased susceptibility to several strains of caliciviruses (3). Influenza virus binding has been shown to vary in relation to host Lewis blood group antigens (89). Further, Raza et al reported that secretor children have increased risk of hospitalization for respiratory infections due to influenza viruses A and B, rhinoviruses, respiratory syncytial virus, and echoviruses (90).

Our own research has demonstrated that susceptibility to ST-associated *E. coli* (27) (6), and several strains of caliciviruses is associated with inhibition by α1,2-linked fucosylated oligosaccharide structures. Our studies with caliciviruses have shown that Norwalk virus-like particles bind to tissue sections of the gastro-duodenal junction from secretors but not from nonsecretors (5), and that binding is blocked by milk from a secretor (91). Volunteers challenged with Norwalk virus become symptomatically infected only if they are secretors. Our laboratory and epidemiologic data suggest that Le^b epitopes and other 2-linked fucosylated oligosaccharide structures inhibit binding by major strains of caliciviruses.

Oligosaccharide synthesis. Most commercially available human milk oligosaccharides are isolated from human milk, are available only in milligram quantities, and are quite expensive. Having been isolated from human material, these oligosaccharides would not be the preferred source for use in humans. Thus, for testing the ability of such oligosaccharides to protect humans from disease, synthetic techniques are required. The classical chemical synthesis of oligosaccharides involves the differential derivatization of the hydroxyl groups of each sugar that is added in sequence to form the desired structure. The hydroxyl group that participates in each linkage must be protected by a different group than the hydroxyl groups that are not involved in linkage. Thus, synthesizing complex structures involves the use of many blocking agents. Over the past 20 years enough blocking agents have been developed to allow the complete chemical synthesis of complex glycan structures that were heretofore not feasible. The advantage of this type of synthesis is that it can be scaled up to make as much sugar as is needed; however, the high price of reagents results in a high cost, even after accounting for economies of scale. There are a handful of contract laboratories that specialize in such syntheses, but the cost of synthesizing a kilogram of trisaccharide ranges from $150,000 to $650,000. A novel chemical approach involves the automated synthesis of these trisaccharides using an array of blocking and de-blocking agents. This approach seems highly promising for synthesizing libraries of oligosaccharides in small quantities, but this technology to date has not been developed for structures containing amino sugars. It is not readily scaled up and for large amounts of oligosaccharide would be more expensive than the classical chemical synthesis.

In the past few years new molecular technologies have been developed for accomplishing some of the critical steps in the synthesis of trisaccharides. In the chemienzymatic strategy, genes for the enzymes that catalyze critical steps toward the formation of essential precursors, and to the reactions that use these precursors to make the desired product are inserted into the appropriate plasmids and transfected into a well-defined bacterium such as *E. coli*. Each *E. coli* transfect overexpresses one key enzyme. The enzyme

is isolated and purified and attached to a solid-phase, which is then packed into a column. The precursors for each reaction are put through the column, and the product is isolated from the eluate. Examples of this technology includes the conversion of the GDP-mannose to GDP-fucose, and the subsequent transfer of the fucose from GDP-fucose onto lactose to form 2′-FL (92). This approach readily allows scale up of the reaction to produce gram- and kilogram quantities of both 2′-FL and 2′-FLNAc. A disadvantage to this approach is the extremely high cost of commercial GDP-mannose. This could be overcome by the use of genetically engineered yeast (*Saccharomyces cerevisiae*). Yeast naturally produce high levels of intracellular GDP-mannose; when the genes for the enzymes that convert GDP-mannose to GDP-fucose are inserted into yeast, the yeast then produces GDP-fucose (93), which can be isolated and purified for use as substrate in the chemienzymatic synthesis of 2′-FL and 2′-FLNAc. The combination of these technologies could be readily scaled up for the large-scale synthesis of oligosaccharides. However, the large number of steps for the isolation and purification of the enzymes for the intermediate and final reactions and for the substrate for the ultimate reaction are somewhat labor intensive. The initial cost for developing this method of synthesis would be relatively high, but the incremental cost for synthesis of additional quantities of product would be more modest. Therefore, this approach has the potential to make human testing of the product economically feasible.

A third approach makes more efficient use of molecular biological techniques: microorganisms are genetically engineered to overexpress substrates and enzymes needed for their conversion into the desired product. In one example, β-galactosidase-negative *E. coli* are grown on a carbon source that results in lactose accumulation within the bacteria. If these bacteria are transfected with genes for the appropriate glycosyltransferases, they will produce specific oligosaccharides characteristic of human milk (94). In another successful example of this approach yeast that have been transfected with genes whose products synthesize the conversion of GDP-mannose to GDP-fucose as described above, are further transfected with genes for the fucosyltransferases; the yeast can then be grown under conditions in which 2′-FL is produced (95). This approach would require the most work to develop, but, when complete, would allow the simple isolation of product from the fermentate to be readily scaled-up for production, with the potential to support the most economical production of quantities needed for human testing.

With the production and isolation of fucosylated trisaccharides these sugars can be tested for their ability to protect against campylobacter and cholera. Furthermore, these sugars can be linked to proteins to produce neoglycoproteins that present the fucosylated epitope in polyvalent form. The inhibitory activity of such neoglycoproteins can be compared with that of the free sugars.

Significance. A growing body of evidence indicates that a critical step in the pathogenesis of campylobacter and other pathogens involves binding to a α1,2-linked fucosylated host cell surface receptor. Human milk α1,2-linked fucosyloligosaccharides would inhibit this binding due to homology between the milk oligosaccharide and the host cell surface receptor. Other related pathogens that share this receptor specificity, eg, cholera, major calicivirus strains, and stable toxin of *E. coli*, would also be inhibited by the same milk oligosaccharides through an analogous mechanism. This inhibition by oligosaccharides could also involve functioning as a chemotactic effector, redirecting campylobacter motility from the mucosal epithelial cells to the lumen of the gut. Our studies to date indicate that 2'-FL alone can inhibit colonization by pathogenic strains of campylobacter. This proposal involves identifying the form of the protective H-2 epitope that is most efficient at inhibiting the target pathogens *in vitro* and *in vivo*, that is, whether the monovalent or polyvalent form will have the highest efficacy. As this is being determined, the most efficient means of synthesizing these protective agents will be tested, so that by the end of the project the most promising inhibitor and its means of synthesis in quantities useful for human testing will be available. Synthetic oligosaccharides that are effective inhibitors of these pathogens could represent a novel and potent class of prophylactic and/or therapeutic agents against campylobacter and several other major enteric pathogens.

Should this supplement be funded, it will (a) increase the amount of free oligosaccharide available for the existing program project, accelerating the progress of the work, (b) allow the active sugar to be tested in its polyvalent form, a form that holds great promise for increased inhibitory activity, and (c) allow new synthetic

techniques to be developed that would significantly decrease the cost and time necessary for synthesis of bulk quantities of the active oligosaccharide or its polyvalent congener. This supplement would thus greatly increase the feasibility of translating our current research into clinical trials that test the efficacy of these sugars as oral agents in secretor individuals and populations that are at increased risk of diarrheal diseases.

C. PROGRESS REPORT

In this section, we outline our major accomplishments and activities, particularly those of the past grant cycle (1998-2003), that most directly pertain to the aims of this supplemental application. We present below evidence from our research that 2'-FL or related structures inhibit binding by campylobacter and cholera and other pathogens, and our capacity and novel schemes for synthesis of active structures in quantities needed for animal, and eventually, human studies. Our data and progress relevant to each of these aspects is described below.

Human milk 2'-FL and other fucosylated oligosaccharides. Using milk samples obtained during weeks 2 - 5 postpartum from 93 Mexican mothers, we measured the concentrations of individual oligosaccharides in each sample. Table 1 shows the results expressed as μmoles per liter of milk and as percentage of the total measured oligosaccharides.

Table 1. Major human milk oligosaccharides

Type	Name	Structure	*μmol/L*	*% OS*
1	LNT, Lacto-*N*-tetraose	Galβ(1→3) GlcNAcβ(1→3) Galβ(1→4) Glc	1273±82	10.1±0.5
1	LNF-I, Lacto-*N*-fucopentaose-I	Fucα(1→2) Galβ(1→3) GlcNAcβ(1→3) Galβ(1→4) Glc	3213±181	25.0±1.1
1	LNF-II, Lacto-*N*-fucopentaose-II	Galβ(1→3)↘ GlcNAcβ(1→3) Galβ(1→4) Glc Fucα(1→4)↗	1149±83	8.9±0.4
2	LNF-III, Lacto-*N*-fucopentaose-III	Galβ(1→4)↘ GlcNA β(1→3) Galβ(1→4) Glc Fucα(1→3)↗		
1	LDFH-I, Lacto-*N*-difucohexaose-I	Fucα(1→2) Galβ(1→3)↘ GlcNAcβ(1→3) Galβ(1→4) Glc Fucα(1→4)↗	1256±104	9.5±0.6
2	LNneoT, Lacto-*N*-neotetraose	Galβ(1→4) GlcNAcβ(1→3) Galβ(1→4) Glc	415±27	3.3±0.2
2	2'-FL, 2'-Fucosyllactose	Fucα(1→2) Galβ(1→4) Glc	3854±108	33.7±1.1
2	3-FL, 3-Fucosyllactose	Galβ(1→4)↘ Glc Fucα(1→3)↗	577±100	4.3±0.5
2	LDFT, Lactodifucotetraose	Fucα(1→2) Galβ(1→4)↘ Glc Fucα(1→3)↗	698±75	5.2±0.4

Mean±SE; *n*=93; LNF-II and LNF-III coelute and are measured together as a single peak. Percent OS equals the concentration of each oligosaccharide divided by the concentrations of all of the oligosaccharides measured x 100. The oligosaccharides measured account for over 90% of the weight of the total oligosaccharides in human milk.

The synthesis pathway of the major milk oligosaccharides is shown in Figure 1.

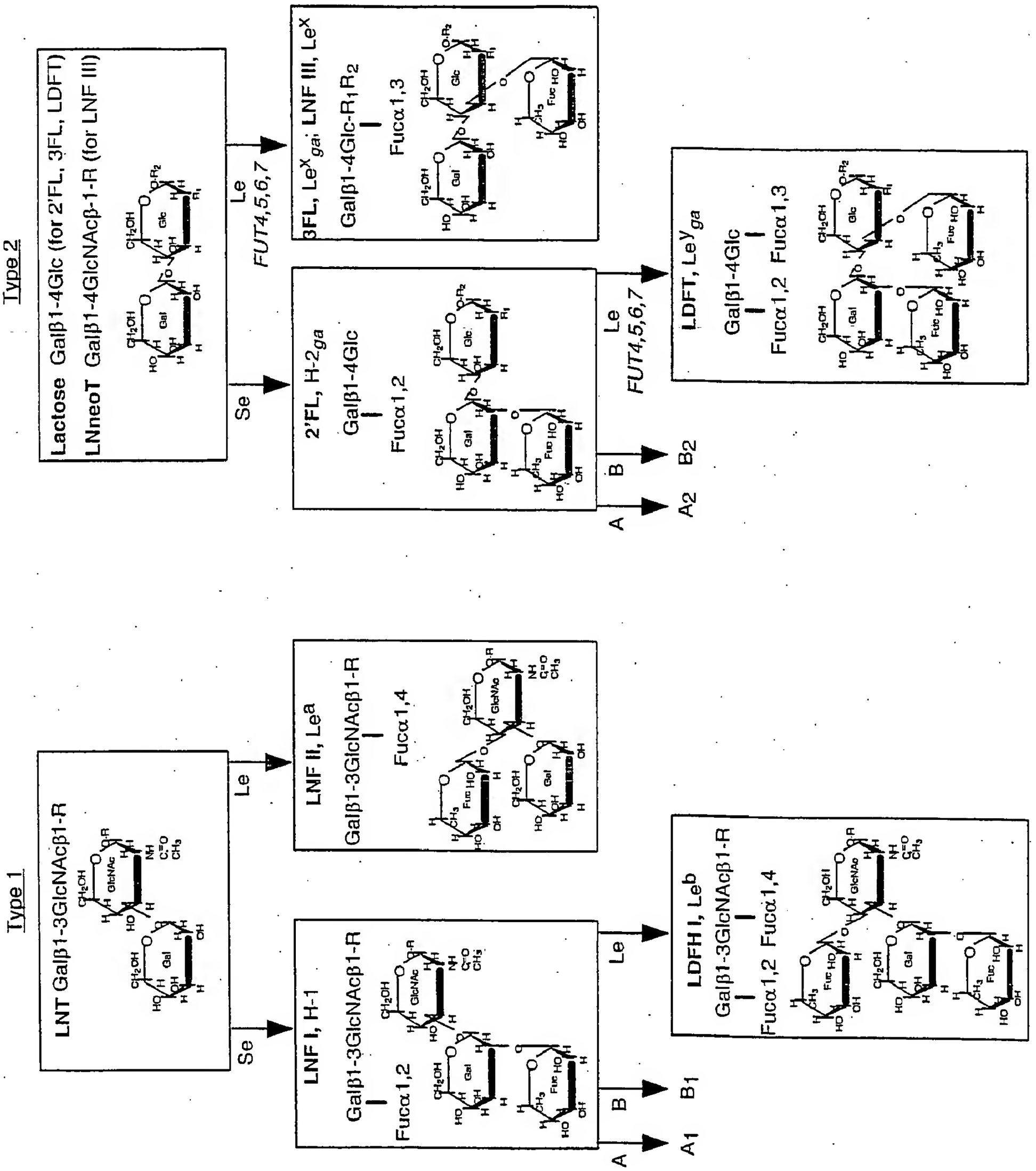
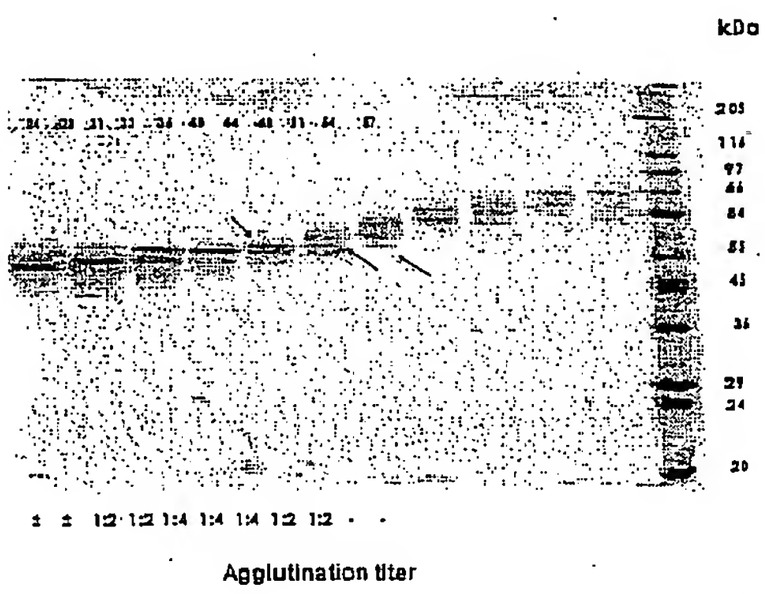
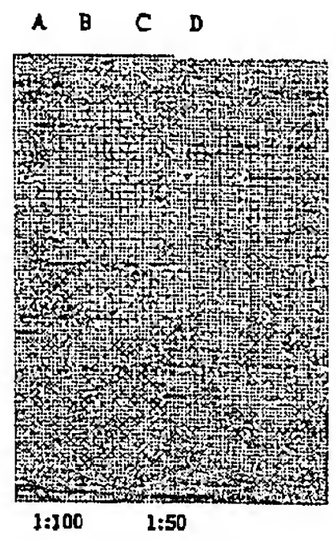


Figure 1. Lewis synthesis pathway applied to human milk oligosaccharide structures. The core type 1 structure, lacto-*N*-tetraose (LNT), is Galβ1,3GlcNAc on the terminal end of lactose (-R). The core for the most abundant type 2 structures in milk includes lactose (for 2'-FL, 3-FL, and LDFT), lacto-*N*-neo-tetraose (for LNneoT), and Galβ1,4GlcNAc on a lactose terminus (for LNF-III). Lewis structural moieties are based on a backbone ending in Gal-GlcNAc; however, the most prevalent type 2 structures in human milk contain lactose (Gal-Glc) and therefore are defined as the glucose analogs (*ga*) to the type 2 Lewis structures, where -R_1 is –OH and -R_2 is –H. True Lewis structures, such as LNF-III, have an R1 of *N*-acetyl and an R2 of lactose or lactosamine. Abbreviations for the fucosyltransferase genes are: Se (secretor gene, *FUT2*), Le (Lewis gene, *FUT3*), and *FUT4*,5,6,7 (Lewis gene family of 3-fucosyltransferases). Blood group A and B structures, synthesized from H-1 and H-2 antigens, have been reported but are not major components of milk oligosaccharides.

Evidence that 2'-FL or related structures inhibit binding by campylobacter and cholera

Identification of campylobacter adhesin. We previously observed that supernatants of *C. jejuni* suspension with deoxycholate induce agglutination of HEp-2 cells. To further characterize this deoxycholate-induced agglutinin, a suspension of a *C. jejuni* culture was incubated with deoxycholate and concentrated by ultracentrifugation. The extract was subjected to continuous-elution electrophoresis, and the fractions were dialyzed and tested in HEp-2 cells agglutination assays (Fig. 2a). The fractions that clearly induced agglutination were those showing a band of 55-57 kDa (Fig. 2b). We are now sequencing this protein.





A , C Crude extract (C. jejuni).
B , D Semipurifided agglutinin

Figure 2a: *Partial purification of the deoxycholate induced Campylobacter jejuni cell agglutinin by continuous-elution electrophoresis. The arrows mark a 55-57 kDa protein with a high cell agglutination activity.*
Figure 2b: *Western blot of the 55-57 kDa agglutinin using a rabbit hyperimmune sera.*

Molecular tools for studying campylobacter-host cell interaction. A major advance during the past funding cycle was the development of molecular tools to further study the interaction of campylobacter with milk oligosaccharides and host cell surface ligands. First, we constructed a shuttle plasmid called pstr008.12 that carries a fluorescent protein (*gfp*) gene of *Aequorea victoria* and a chloramphenicol resistant (*cat*) gene designed to make stable fluorescent constructs that retain the plasmids. Further, we developed plasmids that can be used for expression and site mutation in strains that are multiply antibiotic resistant, a common feature in Mexican strains. This development is important to our work and that of others conducting research in areas of the world with high background rates of antibiotic resistance in pathogenic bacteria.

Characterization of milk receptors that inhibit campylobacter and cholera adherence. During the current grant cycle, we established that the specific binding of campylobacter in HEp2 cells is inhibited by fucosylated carbohydrate moieties containing the H(O) blood group epitope (Fucα1,2Galβ1,4GlcNAc). Studies of campylobacter binding to histo-blood group antigens as neoglycoproteins immobilized in nitro-cellulose membranes demonstrated a high avidity for the H-2 antigen as confirmed by specific inhibition with monoclonal antibodies. In studies on the mechanism of adherence, *C. jejuni*, which normally does not bind to Chinese hamster ovary (CHO) cells, bound avidly when the cells were transfected with a human α1,2-fucosyltransferase gene that caused over-expression of H-2 antigen (Fig. 3). Similarly, *V. cholerae* adheres to transfected (Fig. 4) but not to parental cells (data not shown). This binding was specifically inhibited by H-2 ligands (*Ulex europaeus* lectin, *Lotus tetragonolobus* lectin, and H-2 monoclonal antibody), H-2 mimetics, and human milk oligosaccharides (Fig. 5). Invasive campylobacter 287-IP binds to *FUT1*, but not *FUT3* or *FUT4*-transfected CHO cells (Fig. 6). In experimental models, human milk oligosaccharides inhibited campylobacter colonization in mice *in vivo* and in human intestinal mucosa *ex vivo*. (Fig. 7).

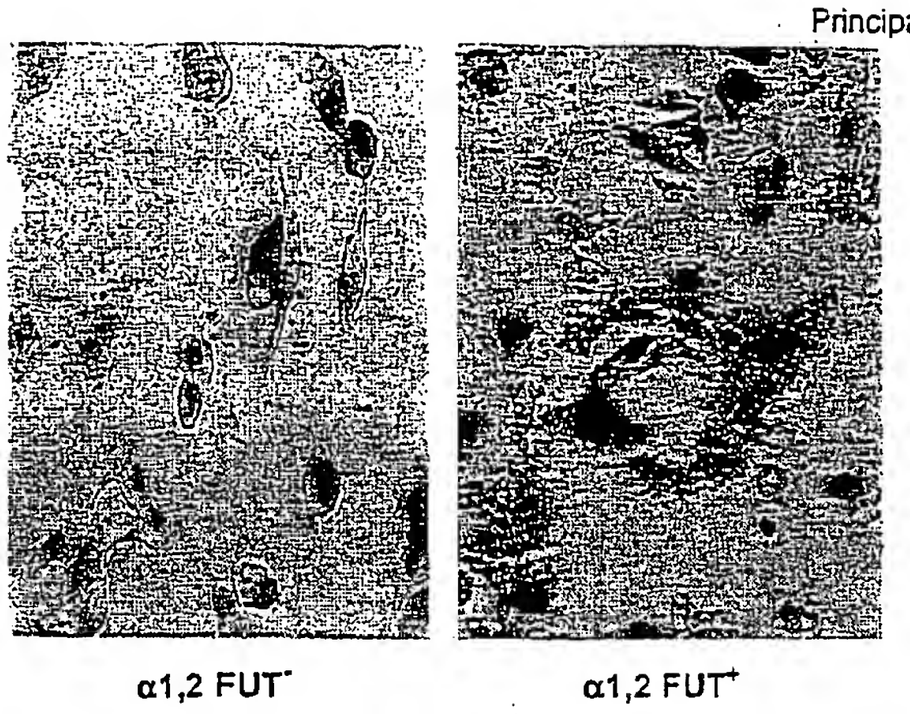


Figure 3: Campylobacter jejuni binding to parental CHO cells carrying the plasmid vector only (right) and binding to FUT1(α1,2 fuc) transfected CHO cells (left).

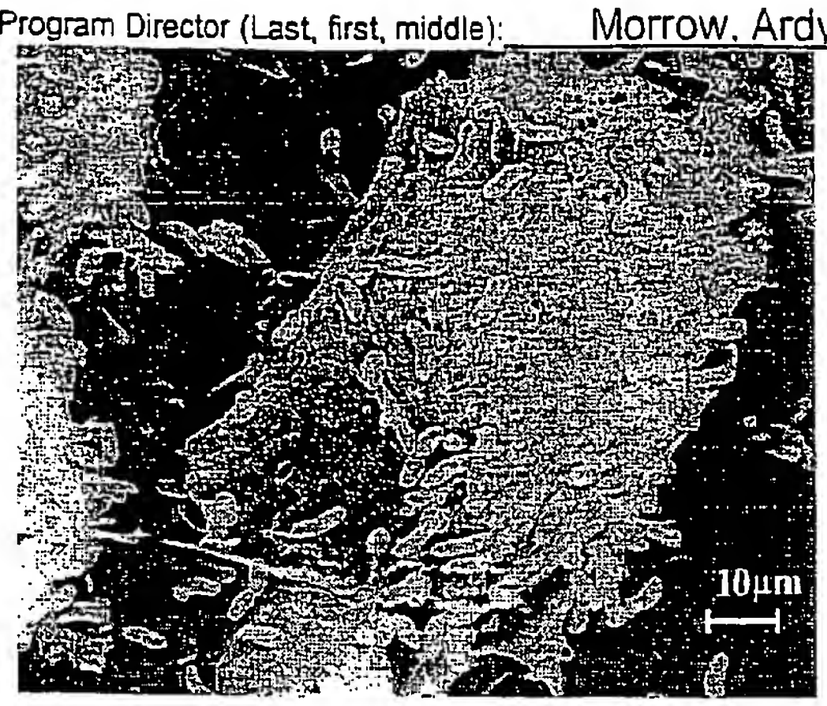


Figure 4: *Scanning microscopy V. cholerae binding to FUT1 transfected CHO cells.*

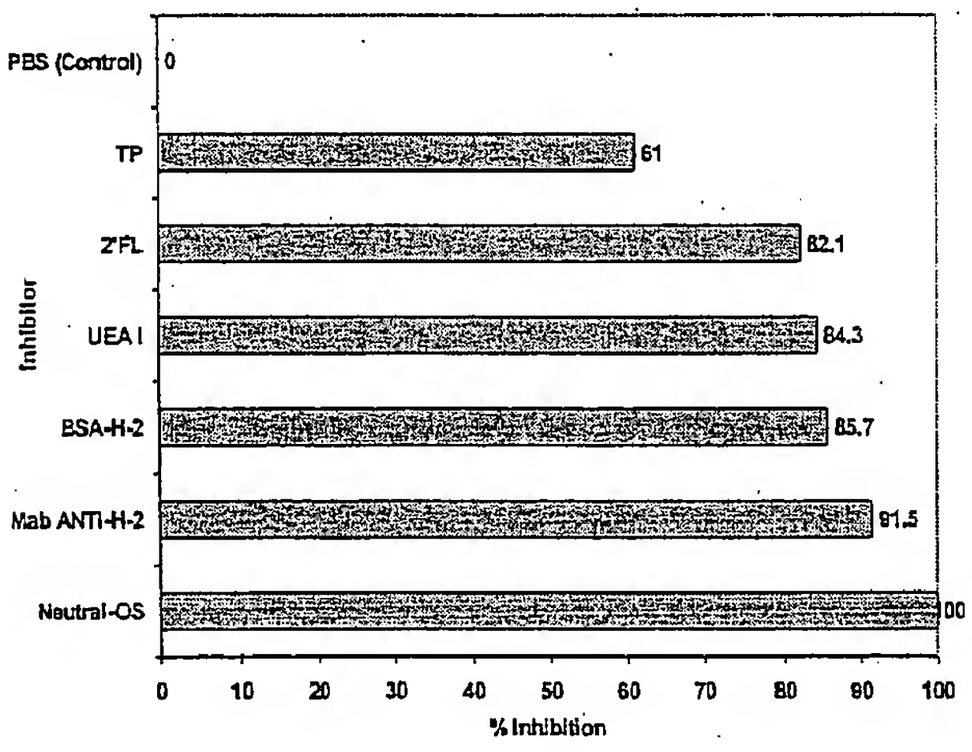
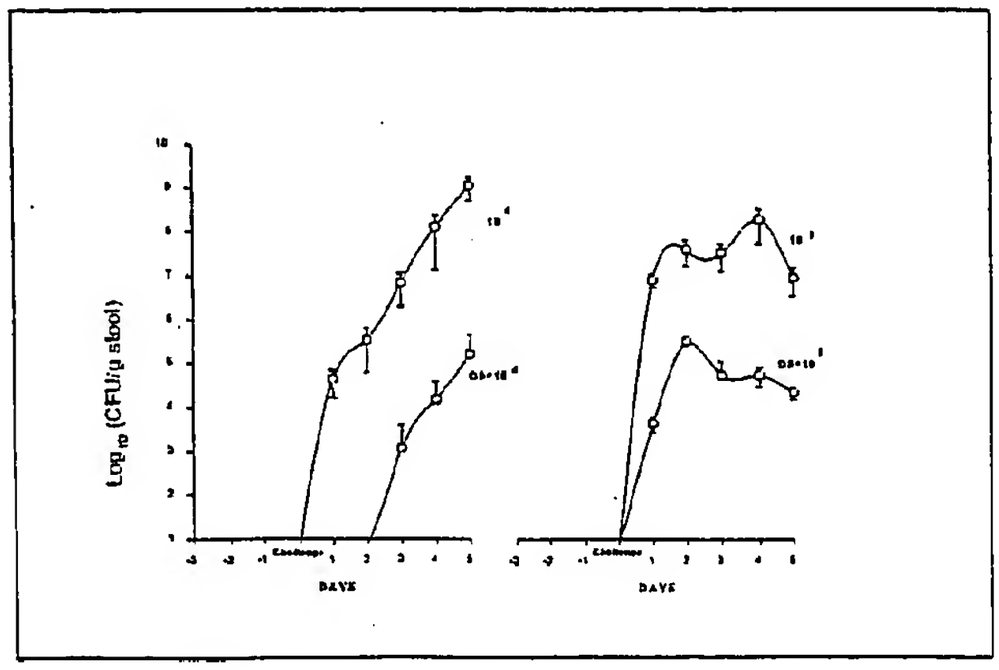


Figure 5: Inhibition of Campylobacter binding to FUT1- CHO cells by H-2 ligands and H-2 mimetics and human milk oligosaccharides.

Cells	Pathogenic					Non Pathogenic	
	UEA I	INN 287IP	INN 84SP	INN 166IP	NN 10SP	INN 50SP	INN 57SP
FUT1 (α1,2)	3+	2+	2+	1+	2+	0	0
FUT3 (α1,3)	0	0	0	0	0	0	0
FUT4 (α1,3 and α1,4)	0	0	0	0	0	0	0
Parental CHO	0	0	0	0	0	0	0

Figure 6: Cell agglutination induced by invasive Campylobacter strain 287ip on transfected CHO cells carrying FUT1 (1,2 fuc), FUT3 (1,3/1,4), and FUT4 (1,3 fuc) gene.



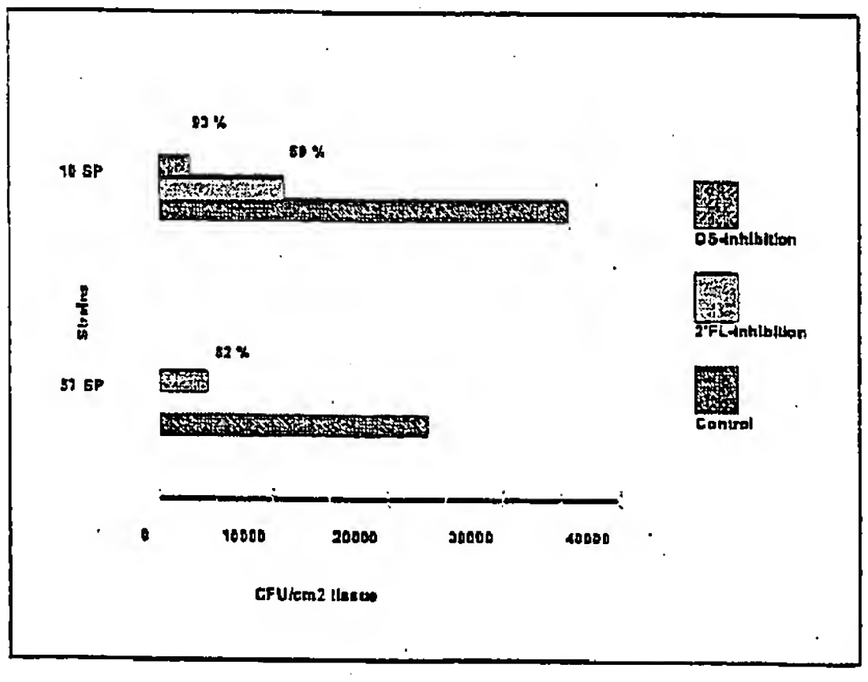
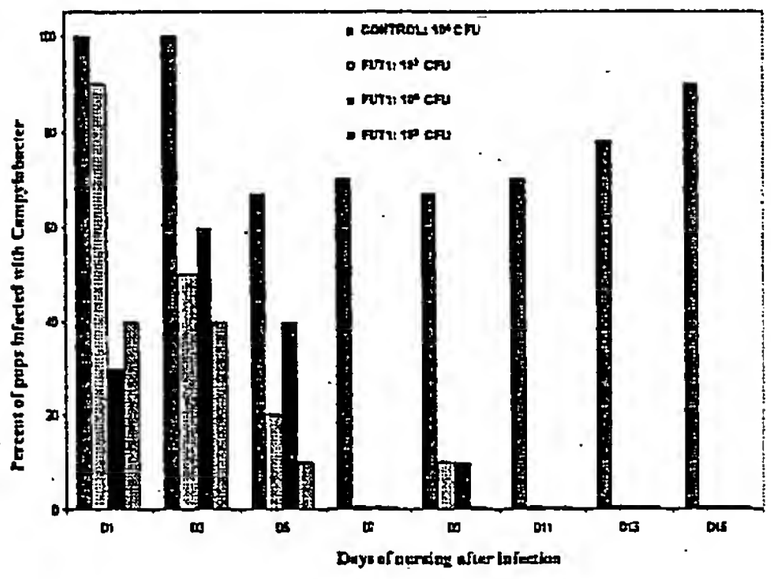


Figure 7: Inhibition of Campylobacter colonization in BALB/c mice fed with 2 mg of milk fucosylated oligosaccharides given during challenge with 10^4 and 10^8 CFU of bacteria (left). Ex vivo assays of inhibition of human gut colonization of Campylobacter with 2'-fucosyllactose (2'-FL) and milk fucosylated oligosaccharides (OS).

Transgenic mice. The role of milk α1,2 glycoconjugates in passive protection against campylobacter infection was evaluated in litters of B6-SJL transgenic female mice carrying the human α1,2-fucosyltransferase gene (*FUT2*) with a whey promoter that induces the expression of histo-blood group antigens primarily in mammary gland during lactation, and thus, in milk. As a control, non-transgenic parental mice were used. Suckling mice were challenged with 10^4, 10^6 and 10^8 CFU of *C. jejuni* and were returned to the dams. Gut colonization was monitored for 15 days. Up to 90% of non-transgenic litters remained colonized during follow-up. Colonization of transgenic mice was transient and the time of colonization was directly related to the inoculum (Fig. 8). These experiments strongly support the role of α1,2-linked fucosylated glycoconjugates of milk in protection against campylobacter infection, and suggest that the main intestinal ligands for campylobacter are the H-2 histo-blood group antigens. Milk fucosyloligosaccharides and specific fucosyl α1,2-linked molecules inhibit this binding. Preliminary experiments of cholera infection in suckling pups from *pWAP FUT1* transgenic dams expressing H(O)



Days after Infection	Transgenic (FUT1)			Non-Transgenic
	Campylobacter inoculum (CFU/ml)			
	$x10^4$	$x10^8$	$X10^9$	Control $x10^8$
1	40	30	90	100
3	40	60	50	100
5	10	40	20	67
7	0	0	0	70
9	0	10	20	67
11	0	0	0	70
13	0	0	0	78
15	0	0	0	88

Figure 8: Campylobacter colonization in transgenic mice carrying the FUT1 gene with the WAP promoter that directs the expression of H antigens primarily to lactating mammary gland. Pups fed from transgenic mice cleared colonization 5 to 9 days after challenge with Campylobacter. Control pups from non-transgenic mice are unable to clear Campylobacter colonization. CFU=colony forming units.

antigen in mammary gland, demonstrated, as with campylobacter, a significant reduction in colonization with an inoculum of 10^8 CFU and a significant reduction in mortality with an inoculum of 10^{10} CFU when compared with non-transgenic controls. Our data strongly support the role of α1,2-fucosyl glycoconjugates in the protection against campylobacter infection and suggest that the main intestinal ligands for campylobacter are the H-2 histo-blood group antigens.

Immunoassays for detection and quantitation of H-2 glycoconjugates in milk. To determine the secretor status of mothers through testing their milk, we developed a Western blot (WB) assay using the lectin *Ulex europaeus* conjugated with peroxidase to detect H-2 glycoconjugates (α1,2-linked structures) (Fig. 9). Milk concentrations of these glycoconjugates were determined by a competitive enzyme-lectin immunoassay (EIA) developed in our laboratory. A curve was standardized with known concentrations of H-2 neoglycoprotein. A total of 139 breast-milk samples obtained from Mexican mothers on day 30 of lactation were tested. A strong correlation was observed between the sensitivities of the two assays to identify non-secretors. In this population, 3/139 (2.2%) were WB- and ELISA-negative, corresponding to non-secretors. The distribution curve of H-2 glycoconjugate concentration in milk is shown in the right hand panel of Fig. 9.

H-2 concentrations in milk differed between individuals; among the 139 Mexican study mothers at least three groups could be identified: 1) 3 (2.2%) non-secretors; 2) 18 (13%) partial secretors, producing less than 5 mg/mL; and 3) 118 (85%) secretors, producing 5 or more mg/mL of 2-linked oligosaccharides.

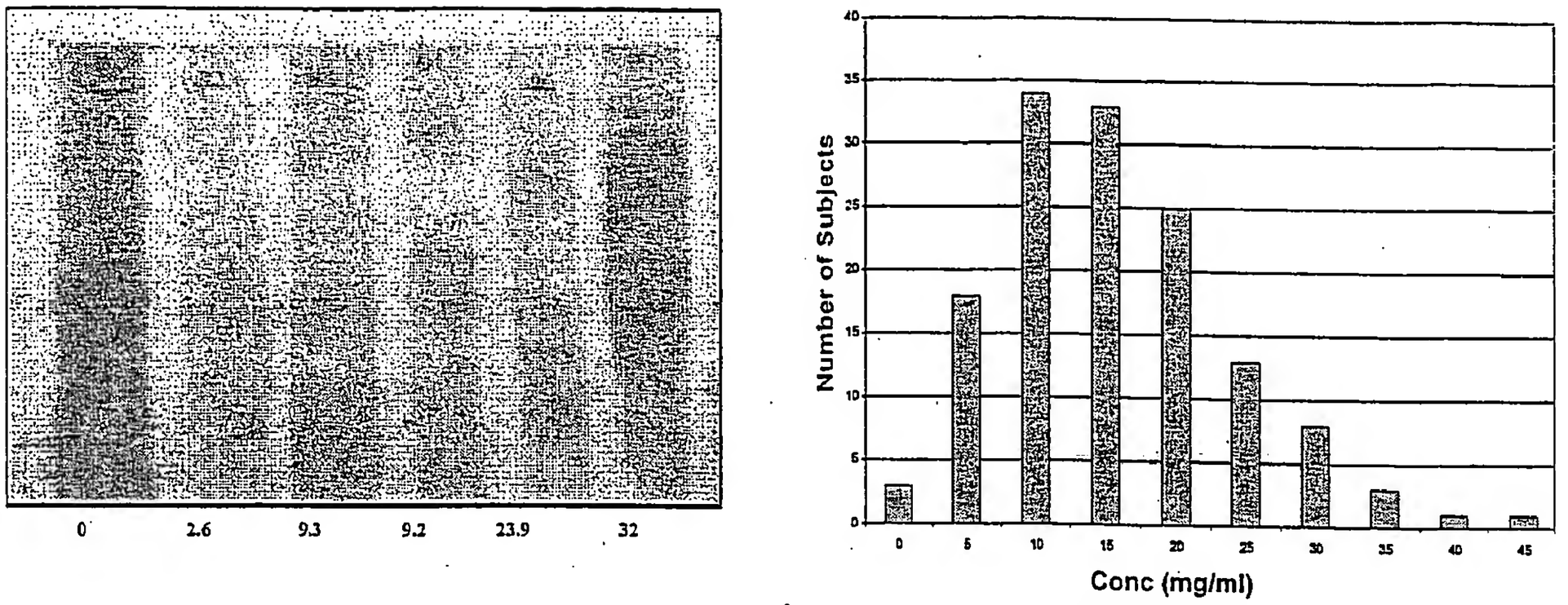


Figure 9: Expression of H antigens in milk. UEA-1 lectin-Western blot of milk samples from Mexican mothers with increasing concentration of α1,2 glycoconjugates (lefthand panel). Concentration of H (α1,2 fuc) antigen in milk (measured as mg/mL) taken at one month postpartum from 139 breastfeeding Mexican mothers as measured by a competitive EIA (righthand panel).

Other enteric pathogens in relation to α1,2-linked human milk oligosaccharides. *Stable Toxin of E. coli.* The seminal early work of this program project was the observation that human milk contained a non-immunoglobulin, low-molecular-weight component, absent from formula or bovine milk (96), that protected suckling mice from ST-induced diarrhea (97). This ST protective factor was isolated and characterized as follows: All of the ST-protective activity was localized to the oligosaccharide fraction and then to the neutral oligosaccharide fraction, specifically in the neutral oligosaccharides that bound to *Ulex europaeus*. This strongly suggested that the ST protective factor was a fucosylated oligosaccharide, because the strongest avidity of *U. europaeus* lectin is for α1,2-linked fucose structures. In tests for biological activity, all fractions were diluted to the volume of milk from which they were isolated; the Ulex-adherent fucosylated oligosaccharide fraction was as effective as human milk itself in preventing ST-induced diarrheal death in suckling mice (6). The mechanism of inhibition was studied in T84 cells, an immortal line of human enterocytes. These cells express guanylate cyclase whose extracellular domain is the receptor for ST and whose intracellular activity produces cyclic GMP, which causes loss of chloride and bicarbonate transport, ultimately leading to the efflux of fluid and electrolytes. In vivo, the result is secretory diarrhea. In the presence of the protective fucosyloligosaccharides of human milk, ST is unable to stimulate production of cyclic GMP, either in intact T84 cells or in isolated membrane preparations. The mechanism of this protection appears to be binding by the oligosaccharide to the T84 extracellular domain of guanylate cyclase, thereby blocking its binding by ST. This prevents the ST-induced loss of chloride ion homeostasis and secretory diarrhea (27). The *U. europaeus*-binding fucosyloligosaccharide fraction was resolved into more than 30 components by semi-preparative HPLC. Of these fractions, only one displayed consistent, robust activity at the concentration found in human milk. This fraction was further subfractionated into seven components of which only one (now identified as a trifucosyl-*iso*-lacto-*N*-octaose – TF*i*LNO) inhibited the diarrheagenic activity of stable toxin in the suckling mouse. We estimate that this subfraction is active at a concentration of approximately 30 ppb, the concentration at which it is found in human milk (98). The total oligosaccharides are approximately 1% of human milk, thus the active TF*i*LNO represents approximately 0.000003% of the oligosaccharides in milk.

Caliciviruses. In collaboration with Dr. Jacques LePendu, Dr. Xi Jiang found that the rabbit hemorrhagic disease virus (RHDV) specifically attaches to rabbit epithelial cells of the upper gastrointestinal and respiratory tracts through the H type 2 histo-blood group oligosaccharide (99). We then tested the ability of NV to recognize such molecules on human gastro-duodenal epithelial cells and found that NV VLPs also bind to such tissue samples derived from individuals of secretor phenotype but not from non-secretor phenotype (5). Non-secretor individuals lack a functional 1,2-fucosyltransferase encoded by the *FUT2*

gene, suggesting that a fucose residue could be involved in the binding. The specificity of NV recognizing these sugar moieties was shown by specific blocking of the binding by human milk from a secretor, by monoclonal antibodies specific for H-1 and H-3 antigens, by synthetic oligosaccharide conjugates containing secretor antigen, and by treatment of the tissues with α1,2 fucosylsidase (5). NV also binds to differentiated CaCo2 cells and it is known that differentiated CaCo2 cells express human histo-blood group antigens (5). Transfection of Chinese Hamster Ovary (CHO) cells with an α1,2 -linked fucosyl-transferase cDNA allowed attachment of NV VLPs (5).

We then performed a study to determine if different strains of CVs have the same host-specificity as that of NV. We tested saliva samples from 51 volunteers for binding eight recombinant capsid antigens representing seven genetic clusters of CVs with EIAs (100). The phenotypes of histo-blood group antigens of the 51 individuals were determined by EIA using monoclonal antibodies specific to Le^a, Le^b, H type 1, and A and B blood group antigens. The eight strains of caliciviruses revealed four patterns of binding among associated with differing histo-blood group types. The saliva samples were boiled before being tested to inactivate antibody (100). Three patterns (strains 387, NV and MOH) bind secretors and one pattern (strain 207) reacts with both non-secretors and secretors but prefers non-secretors. The three secretor-binding strains can be further divided based on the ABO types: 387 recognizes all secretors (A, B and O), NV recognizes A and O, and MOH recognizes A and B.

To investigate the association between secretor status and susceptibility to NV infection, Dr. Jiang collaborated with Dr. Christine Moe in Emory University School of Medicine on a volunteer challenge study. Saliva samples from 50 NV-challenged volunteers were tested for secretor status and for NV-binding (101). There was a strong association between secretor status and binding ($p<0.000$) with 68% of secretors and 76% of these also binding NV capsids. Only 6% of the saliva samples from the non-secretors bound NV capsids. Secretors were almost 40 times more likely to become infected with NV than non-secretors, strongly suggesting that susceptibility to NV infection depends on secretor status. Following the discovery of CV receptors in intestinal epithelial cells and saliva, Dr. Jiang characterized human milk samples for their levels of activity in blocking CV binding to human saliva. A total of 60 milk samples from 30 Mexican mothers and 30 US mothers were studied using the most dominant strain of CV (VA387, genogroup II). Significant blocking activities (30-90% decrease in OD) were observed in 54 (90%) of the 60 mothers (100). This was not associated with antibody because the milk samples were boiled before testing to inactivate antibody. In conclusion, human milk contains elements that specifically block CV binding to histo-blood group antigen receptors, which may provide protection to infants from CV infection.

Protection of breastfed children associated with human milk α1,2-linked fucosyloligosaccharides. As part of the program project grant activities we selected a subgroup of 93 breastfeeding mother-infant pairs from the 1988-1992 cohort conducted in Mexico City to examine protection of breastfeeding children against campylobacter, calicivirus and all causes of diarrhea in relation to variation in quantity α1,2-linked fucosylated oligosaccharides found in human milk. The data from these mother-infant pairs included the follow-up time from birth to the end of breastfeeding. A single milk sample from each of these mothers, collected at 1-5 weeks postpartum, was analyzed by HPLC in Dr. Newburg's laboratory. The profile of milk oligosaccharides was examined in relation to risk of infant diarrhea due to campylobacter. Of the 93 study children, there were 31 cases of symptomatic campylobacter infections and 16 cases of symptomatic calicivirus infections during the breastfeeding period. The fucosylated milk oligosaccharide phenotype differed by maternal Lewis blood group type. The most common milk oligosaccharide in all study mothers was 2′-FL, the oligosaccharide homolog of the H-2 epitope. The mean concentration of 2′-FL in maternal milk samples was 3854±108 nmol/mL (34% of total fucosylated oligosaccharides). Only 2′-FL as a percentage of total oligosaccharide was significantly associated with protection against campylobacter diarrhea (Poisson regression β coefficient=-5.6 [SE=1.9], p=.004). Protection against calicivirus diarrhea was significantly associated only with LDFH-I, the oligosaccharide homolog of the Le^b epitope (Poisson regression β coefficient=-13.3 [SE=5.3], p=0.012). 2-linked oligosaccharide as a percent of total oligosaccharide was significantly ($p<0.001$) associated with protection against mild to moderate diarrhea of all causes (figure 10). This manuscript has been submitted for publication and is currently in review; these data have also been presented at several national and international meetings (102).

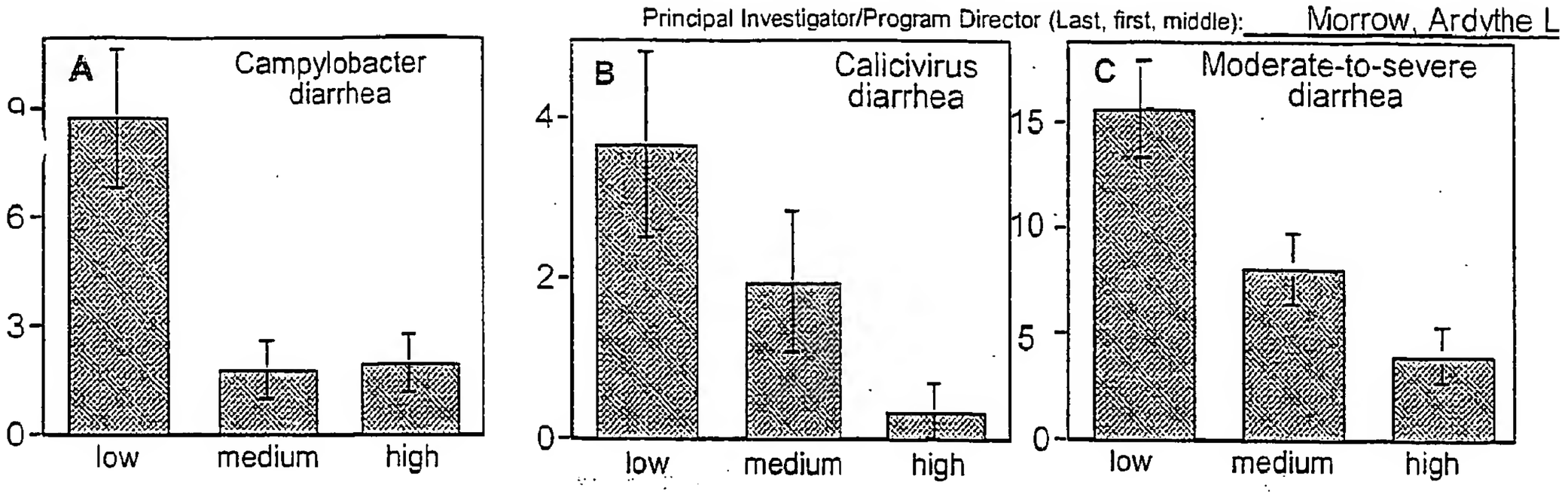


Figure 10. The incidence of *C. jejuni diarrhea*, calicivirus diarrhea and moderate to severe diarrhea of all causes in study children whose mother's milk contains low, medium, or high relative amounts of **(Panel A)** 2'-FL, **(Panel B)** LDFH-I, and **(Panel C)** total 2-linked fucosylated oligosaccharide as a percent of milk oligosaccharide. The bars indicate the cause-specific incidence rates of diarrhea in each group; the vertical lines indicate the standard error. The low, medium, and high groups each represent the oligosaccharide values of a tertile (n=31) of the study population. **Panel A**: For 2'-FL, the percent of milk oligosaccharide values by group: low (<0.29), medium (0.29–0.36), and high (>0.37). Compared to the low group, campylobacter incidence in the medium and high groups were both significantly (P<0.01) reduced. **Panel B**: For LDFH-I, the percent of milk oligosaccharide values by group: low (<0.07), medium (0.07–0.11), and high (>0.12). Compared to the low group, calicivirus incidence in the high group was significantly (P=0.02) reduced. **Panel C**: For total 2-linked fucosylated oligosaccharide, the percent of milk oligosaccharide values in each group: low (<0.72), medium (0.72 – 0.77) and high (>0.77). Compared to the low group, incidence of moderate-to-severe diarrhea in the medium and high groups were both significantly (P<0.01) reduced.

Capacity for synthesis of relevant structures. The human oligosaccharide structures that we wish to test in animal models are available commercially, but they are derived from human milk, and are not affordable in the quantities required. Thus, a critical component of our Program Project and this supplemental application is our approach to ensuring appropriate synthesis of adequate quantities of the putative protective structures for testing in animal, and eventually, human studies. Three major strategies for synthesis of oligosaccharides are proposed: purely chemical techniques, using existing and well established technology, chemienzymatic techniques, with recombinant enzymes produced via gene transfection of microorganisms, and total synthesis by genetically engineered organisms. Each of these approaches has advantages and disadvantages. The most secure technologies are the most expensive for large-scale synthesis but the use of these older technologies allows us to test the efficacy of our most promising structures in animals, allowing us to focus our synthesis on a specific structure. Developing technologies and testing new strategies for synthesis of the active oligosaccharide will allow production of amounts that are suitable for human studies at costs that will allow a range of doses, adequate length of studies, and optimal sample size. This is needed so that the human studies that will follow this project are able to provide definitive results.

We have interacted with a number of companies and synthetic chemists to determine the best approaches to meeting our scientific aims in an affordable and timely manner. To support the proposed animal studies, our first choice of synthesis method is chemical because it is the most secure. Synthetic chemists at Alberta Research Council have the necessary expertise and experience in large-scale synthesis of this type of oligosaccharide, and can supply the quantities of 2'-FL required for animal studies at the lowest possible cost for this type of synthesis (See letter, Appendix 1). For the most active epitope(s) we anticipate that synthesis must be further scaled up to achieve the multi-kilogram quantities necessary for human testing. Unfortunately, the unit cost for structures manufactured chemically would not be significantly reduced through increasing the volume synthesized. Thus, while using oligosaccharides made by chemical synthesis for testing the most promising structures for their ability to inhibit pathogen colonization in animals, we would concurrently develop a molecular approach to synthesis based on genetically altered microorganisms that are readily scaled up for economical large-scale synthesis. In one approach, organisms are induced to overexpress enzymes needed for each step of the synthesis. This approach could use GDP-fucose made by a recombinant yeast. In another approach, recombinant yeast that produce GDP-fucose could be further

transfected with fucosyltransferases so that the 2'-FL or 2'-FLNAc can be synthesized by these yeast, and the product isolated from the incubation medium. We anticipate that these techniques will lead to more flexible and efficient large-scale synthesis of a variety of protective structures; in all cases the final steps of purification and quality assurance would place in a GMP laboratory. Detailed descriptions of these methods are found in the Experimental Design and Methods section below.

Summary of our progress. We have made significant strides over the past five years in characterizing the oligosaccharide structure(s) that may be associated with inhibition of campylobacter and cholera binding to host ligands. We have also identified effective approaches to synthesis that will allow animal testing of putative protective structures and novel synthesis approaches that could translate into human testing to be conducted upon completion of the research described in this supplemental application.

D. EXPERIMENTAL DESIGN AND METHODS

The following section represents the experimental design and methods that would be used over a four year period to fulfill the three specific aims of this supplemental request. In the current year of the parent grant, we plan to determine, through in vitro assays, whether the human milk oligosaccharide, 2'-FL, or its homolog 2'-FLNAc, is the more effective inhibitor of binding by campylobacter and by cholera. The oligosaccharides to be tested in these *in vitro* assays are unconjugated, and required in small quantities, and can thus be procured from commercial sources. This program project grant aim will be completed in the laboratory of Dr. Ruiz-Palacios this year, and is noted here because it is preparatory to specific aim 1 of this supplement, as indicated below.

Design

Specific Aim 1. Synthesize polyvalent forms of 2'-FL, a human milk trisaccharide, and determine if inhibition of binding by campylobacter and by cholera *in vitro* are enhanced.
The trisaccharide that proves to be the most active in the in vitro assays being undertaken in the program project grant this year, either 2'-FL or 2'-FLNAc, will be synthesized in polyvalent forms as neoglycoproteins. Different densities of sugar moieties bound to the protein backbone, different types and sizes of spacers connecting the trisaccharide to the backbone, and different backbone proteins will be investigated. The strength of inhibition afforded by each of these structures will be compared with each other and with the free parent oligosaccharide using both solid phase and cell culture assays described above. To fulfill this aim, we will determine whether neoglycoproteins containing any of several different densities, spacers and backbones are more effective inhibitors of binding by campylobacter and by *V. cholera in vitro* than the simple parent trisaccharide.

Specific Aim 2. Test the most promising anti-campylobacter and anti-cholera oligosaccharide in animal models.
Two of the oligosaccharides most active *in vitro* will be tested for activity against colonization of mice by campylobacter and *V. cholerae*. The oligosaccharides to be tested will be the free trisaccharide and the neoglycoprotein with the strongest inhibitory activity. Two models will be used for each infection: one is a model for the efficacy of the carbohydrate as a prophylactic agent against colonization, and the other for use of the agent as a treatment after colonization. The complexity of the intact intestine relative to in vitro assays may influence the relative efficacy of the polyvalent relative to the free monomeric oligosaccharide in unpredictable ways. If the results in vivo confirm the results in vitro, the active oligosaccharide or neoglycoconjugate will be ready for human studies. The relative protection prophylactically and post colonization will allow the optimum design for testing the agent in humans. The parent grant already supports animal testing to determine the efficacy of the 2'-FL or 2'-FLNAc trisaccharide, however, the ability to conduct those animal studies is dependent on the chemical synthesis proposed in this supplement. The parent grant does not support animal testing of promising neoglycoprotein structures, which would be made possible by this supplement.

Specific Aim 3. Investigate novel routes for the efficient synthesis of large quantities of the protective compound.

One of the few laboratories capable and willing to synthesize kilogram quantities of 2′-FL or 2′-FLNAc has committed to manufacture enough of the active compound to allow us to complete the proposed animal challenge studies within the constraints of the proposed budget. Concurrent with these studies, we will explore and develop a method for the synthesis of the active oligosaccharide through chemienzymatic synthesis scheme and through the construction of genetically modified yeast. Analogous technology will be used to synthesize a neoglycoconjugate that contains the active oligosaccharide epitope attached to a backbone of protein and complex sugar spacers that closely resemble those found in human milk. Such novel synthetic schemes would allow us to produce quantities of protective molecules sufficient for clinical trials in a cost effective manner. Thus, the completion of this proposed research would permit planning for practical larger-scale human trials.

Specific Methods

1) Synthesis of oligosaccharides and neoglycoconjugates

The oligosaccharides required for our current *in vitro* experiments require relatively small amounts that can be procured from conventional commercial sources, however, the oligosaccharides that we propose to test in animal models in our program project (2'-FL or 2'-FLNAc) are not affordable from conventional commercial sources in the quantities required. Most commercially available human milk oligosaccharides are isolated from human milk. They are available only in milligram quantities, are quite expensive, and, having been isolated from human material, would not be the preferred source for use in humans. Thus for the proposed animal studies in our parent grant and in preparation for human trials, we will undertake synthesis of the necessary oligosaccharide structures and their neoglycoconjugates.

Three major strategies for synthesis of oligosaccharides are proposed: purely chemical techniques, using existing and well established technology, chemienzymatic techniques, with recombinant enzymes produced via gene transfection into microorganisms, and total synthesis by genetically engineered organisms (see Fig. 11, 12, 13). Each of these approaches has advantages and disadvantages. The most secure technologies are the most expensive for large-scale synthesis. However, such classical chemical synthesis can be used to test our most promising structures in animals, to define the most active of the forms in which the active epitope may be presented *in vivo*. While this is occurring, we will be developing techniques that would allow synthesis in amounts that are suitable for human studies at costs that will allow appropriate dosages, optimal sample size, and duration of studies. This would allow the clinical trials that are anticipated to follow this project to have their greatest chance to provide definitive results.

Conventional chemical synthesis. For the amounts needed for testing in animals, we have a letter of understanding with the Alberta Research Council for the chemical synthesis of kilogram quantities of the trisaccharide of interest at high purity suitable for animal studies. The classical chemical synthesis of oligosaccharides involves the differential derivatization of the hydroxyl groups of each sugar so that they can be joined chemically in the precise sequence required to form the desired structure. The hydroxyl group that participates in each linkage must be protected by a different group than the hydroxyl groups that are not to be involved in linkage. Our strategy for this chemical synthesis is as follows:

The choice of glycosyl donors and acceptors, protective group strategies, and coupling conditions for formation of α-L-fucosyl (*cis*), and β-D-galactopyranosyl (*trans*) linkages will be according to the established methods of modern synthetic carbohydrate chemistry (103-105). The reactions will be "conventional" in the sense that the objective is to obtain the compounds as quickly as possible in good yield, rather than to explore novel chemistry (Fig. 11).

Glycosyl donor for α-L-fucosyl residue. The critical requirement is for a donor with a "non-participating" group at O-2 (106). For this purpose 2,3,4-tri-O-benzyl-α-L-fucopyranosyl bromide will be employed, under conditions of halide ion catalysis, a method that has been successfully employed in many syntheses of α-L-

fucosyl derivatives (107-111). The use of benzyl ether groups as persistent protective groups (112,113) will have the additional advantage that after coupling, deprotection of donor and acceptor residues in the target compounds can be achieved at the same time by catalytic hydrogenolysis. The use of alternative donors, such as 1-thio, fluoride, trichloroacetimidate, or 4-pentenyl glycosides (114) will only be considered if the above approach is found to be unsatisfactory.

2-Acetamido-2-deoxy-D-glucopyranose acceptors (GlcNAc acceptors). Initially, the compounds will be benzyl, 4,6-benzylidene, or allyl ether derivatives of benzyl glycosides (see figure), but the groups will be manipulated so that primarily benzyl ethers will remain at the end of the synthesis. A final step of catalytic hydrogenolysis will deprotect residues derived from both acceptor and fucosyl donor. The regioselectivity of the reductive ring opening of benzyl 2-acetamido-4,6-O-benzylidene-2-deoxy-D-glucopyranoside derivatives with lithium aluminum hydride/aluminum chloride is dependant on the steric bulk of the substituent at O-3 (115).

β-D-Galactopyranosyl donor. This requires strict protective group requirements. The primary requirement is for a "participating" group at O-2 (105). At O-1 a temporary protective group must allow introduction of a halogen atom as a leaving group in the glycosidation reaction. Bromine is preferable because of its greater reactivity (105), and the introduction should be under the mildest conditions practical. Therefore the O-1 substituent will normally be p-nitrobenzoyl, to be reacted with hydrogen bromide in dichloromethane (116). At O-2, a "participating" group is necessary for 1,2-*trans* -glycoside formation. O-benzoyl is favored over O-acetyl because the O-benzoyl group is less labile to basic conditions, less prone to migration, less likely to undergo unwanted ortho ester formation during coupling reactions, and easier to introduce selectively (112). At the O-2 position where linkage is desired for 2'-FL and 2'-FLNAc, a temporary protective group is necessary that can be removed after the first glycosidation without affecting other linkages or groups; the benzoyl group will perform both functions. The remaining two positions must be occupied by persistent groups (benzyl), to be removed only at the end of the synthesis.

The coupling reaction between the specially protected "internal" galactosyl donor and the protected glucosamine acceptor will employ silver triflate as promoter, in the presence of acid scavengers (collidine or tetramethylurea), and molecular sieves, i.e., standard conditions for *trans* glycoside coupling (117-119). Should this prove unsatisfactory (e.g., because of a lack of strict stereospecificity) Helferich conditions (120) (mercuric cyanide/mercuric bromide promoter) or Koenigs-Knorr conditions (121) (silver carbonate as insoluble catalyst) can be substituted. Whichever method is used, after chromatographic purification of the product of the first glycosidation reaction, it may be necessary to perbenzylate to avoid any risk of intermolecular acetyl migration during the subsequent fucosylation step. However, if experimentation indicates that this is not a problem, the perbenzylation step will be left out.

Starting compounds, chromatography, deprotection, and structure confirmation. All the starting materials are accessible from L-fucose, D-galactose, and 2-acetamido-2-deoxy-D-glucose, via procedures described in figure 11. Their preparation and the coupling reactions will be followed by TLC to monitor the purification of the intermediates. The products of coupling reactions will be purified by silica gel column and preparative layer chromatography. Deprotection steps are expected to be straightforward as a result of careful choice of the groups to be used. GlcNAc acceptors are shown as benzyl glycosides for simplicity, though 2-bromoethyl glycosides or 8-methoxycarbonyloctyl glycosides, for example, may be substituted if necessary for protein conjugation. The synthesis of 2'-fucosyl-N-acetyllactosamine (110) has been described, providing experimental and spectroscopic data for preparing intermediates and characterizing products. Purity of final products will be determined by TLC and HPLC, and a final purification by passage through a column of Bio-gel P-2 and/or a coupled column of cation and anion-exchange resins will be performed when necessary. Structures of key intermediates and final products will be confirmed by permethylation analysis and mass-spectrometry.

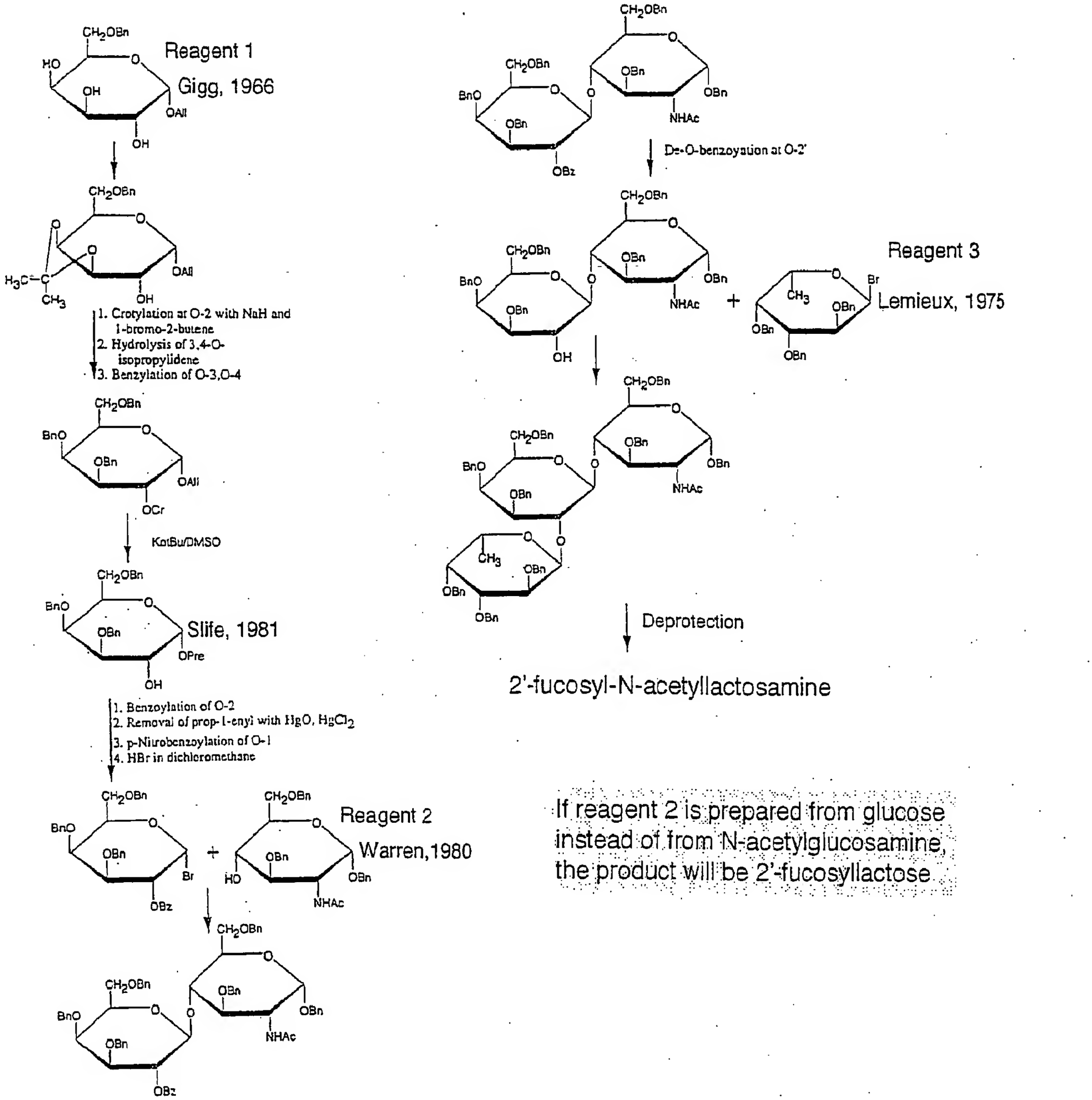
This synthesis has the least need for development of novel synthetic steps, and can predictably be synthesized for animal testing while other methods of synthesis are being developed, as follows:

Chemienzymatic synthesis. *Synthesis of GDP-fucose.* GDP- fucose from commercial sources is not available in quantities and at prices that could be used as chemienzymatic synthesis of these oligosaccharides and their conjuners. Therefore, the first step of chemienzymatic synthesis will be the production of GDP-fucose through its production in genetically engineered yeast, Saccharomyces cerevisiae. A major metabolic product of wild-type S. cerevisiae is GDP-mannose. In most higher organisms GDP-mannose is the precursor of GDP-fucose through the action of enzymes: GDP-D-mannose-4,6 dehydratase, synthesized in E. coli by the gmd-gene and GDP-4-keto-6-deoxy-D-mannose epimerase/reductase, which is synthesized by the wcaG-gene. This construct has been transformed into yeast as the pESC-leu/gmd/wcaG vector. In order to induce GDP-fucose synthesis in S. cerevisiae the E. coli gmd-gene and wcaG-gene, i.e., GMER (FX) were inserted into pESC-leu-vector under GAL1 and GAL10 promoters, respectively. The gmd was inframe with c-myc-epitope and the wcaG with FLAG-epitope. This transfect of S. cerevisiae produces approximately 0.2 mg/L of GDP-fucose without addition of any external GDP-mannose (93). We will refer to this yeast mutant as GDPF-sc (Fig. 12).

Synthesis of 2'-FL and 2'-FLNAc. The synthesis of 2'-FL and 2'-FLNAc will be carried out using 2'-FL enzymes expressed in *E. coli.* These will be expressed from fucosyltransferase genes, either human (*FUT1* and *FUT2) or from Helicobacter pylori (FucT2).* Human *FUT1* and *FUT2* are currently in use in our laboratory for the transfection of CHO cells. These genes will be inserted into *E. coli* an appropriate vector such as pGEX4T-1, and overexpressed. The fusion protein will be purified by affinity chromatography on a GSTrap-column (Pharmacia/Amersham Biosciences). The purified fucosyltransferase enzyme will be covalently linked to sepharose through the solid phase reductive amidation. To columns packed with this sepharose-bound fucosyltransferaewe we will add GDP-fucose and lactose in PBS to produce 2'-FL or GDP-fucose. GDP-fucose and N-acetylactosamine will be run through the column to produce 2'-FLNAc. Excess GDP-fuc and nucleotide phosphate will be removed by ion exchange. The fucosylated oligosaccharides will be separated from their starting materials by passing them through a *Ulex europaeus* affinity column. The yield and purity of these products will be assessed by HPLC analysis of the resulting oligosaccharides.

Synthesis of polyvalent neoglycoproteins. Two approaches will be used for the synthesis of neoglycoproteins of these sugars. The first utilizes the techniques described above for the synthesis of neoglycoproteins from BSA. The second approach will utilize neoglycoproteins synthesized in yeast by GlycoFi (Lebanon, NH). The non-fucosylated neoglycoprotein in solution with GDP-fucose, as above, will be passed through the solid-phase fucosyltransferase column. GDP-fucose and sugar will be removed from the eluate through ion exchange. Fucosylated neoglycoprotein will be separated from the non-fucosylated starting material through its binding to a *U. europaeus* affinity column.

Synthesis by genetically engineered yeast. Wild type yeast will be transformed into GDPF-sc mutants to produce GDP-fucose through the introduction of gmd and wcaG genes. These GDPF-sc yeasts will be further transformed to incorporate fucosyltransferase into the yeast cell wall. This is accomplished through the fusion of the fucosytransferase with the yeast cell wall protein Pir. The plasmid is constructed such that the fucosyltransferase will be attached to Pir through the N-terminal region so that the expressed transferase will be found in the extracellular side of the yeast cell wall. A fusion gene will be constructed by fusing either the transmembrane domain truncated FucT2 from *H. pylori* or the human gene, FUT2 to the Pir1 gene of S. cerevisiae through the HA epitope, tag. This fusion gene will be inserted into a multicopy plasmid YEp352GAP-2 expressing under the control of S. cerevisiae GAPDH (glyceraldehydes-3-phosphate dehydrogenase) promoter. We will designate these yeast mutants as FUT-sc. These FUT-sc transformed yeasts, when provided an excess of lactose, are expected to produce 2'-FL. After centrifugation



If reagent 2 is prepared from glucose instead of from N-acetylglucosamine, the product will be 2'-fucosyllactose

Figure 11. Chemical synthesis of 2'-fucosyllactose and of 2'-fucosyl-N-acetyllactosamine

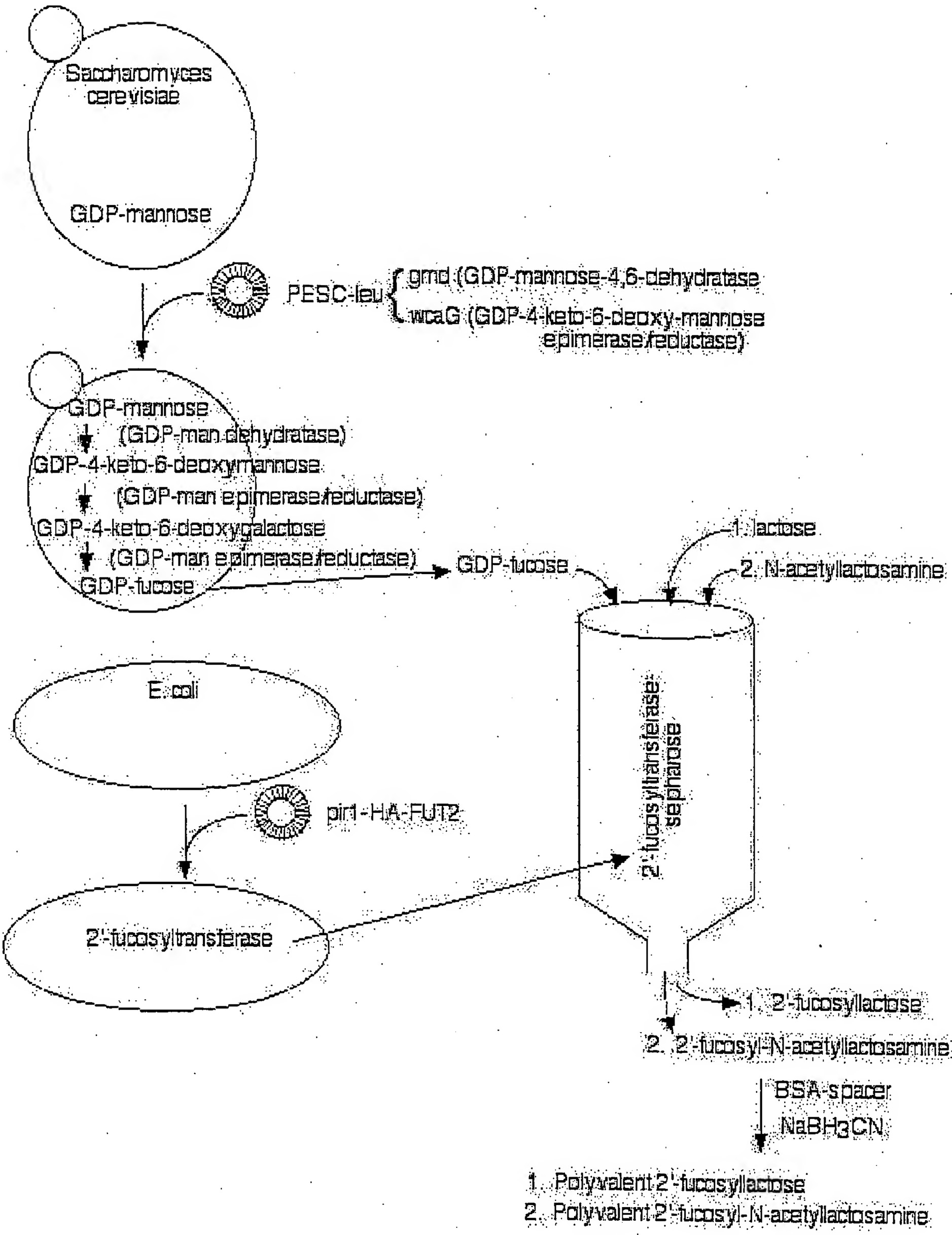


Figure 12. Chemienzymatic Schema

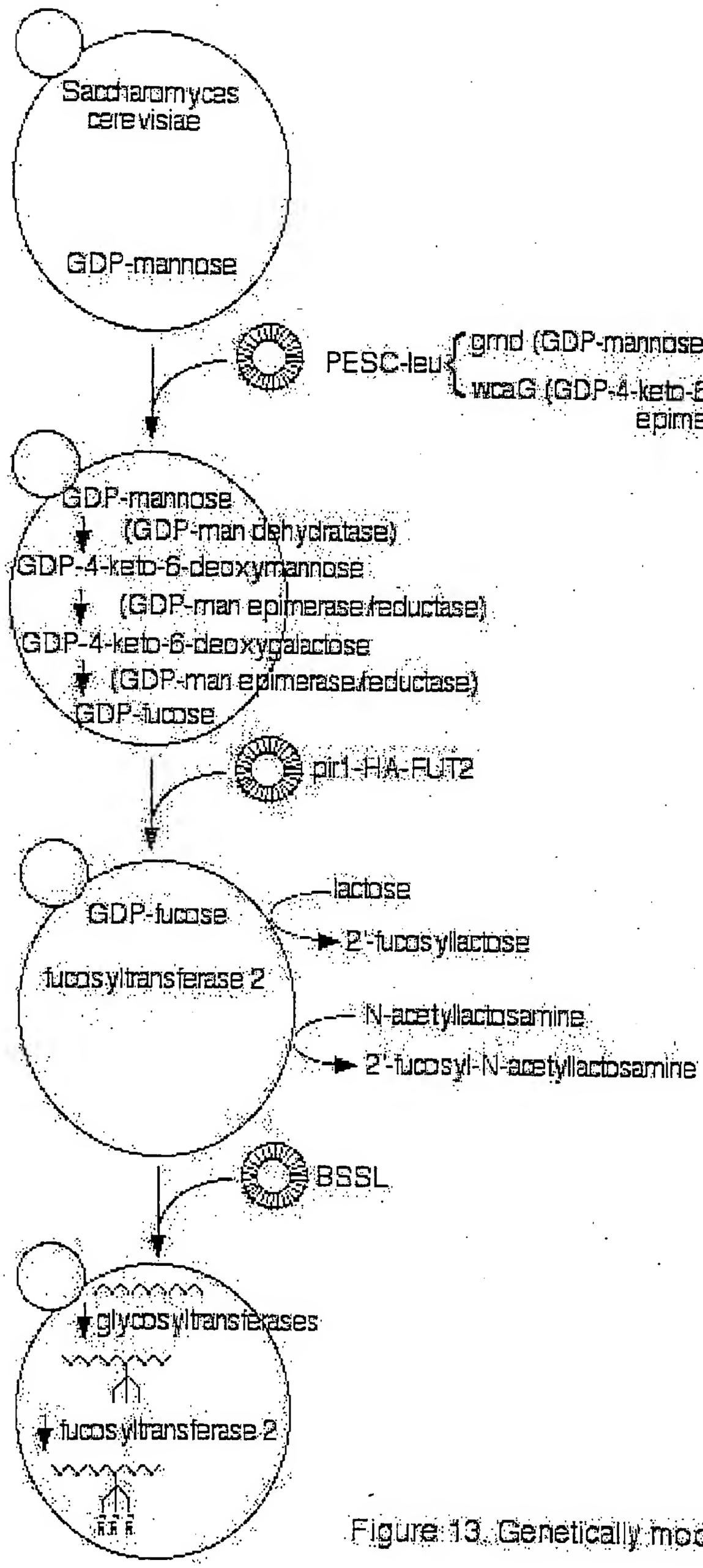


Figure 13. Genetically modified yeast synthesis schema

and filtration this product will be isolated by its avidity to a Ulex lectin affinity column. Similarly, when these transfected yeasts are provided the substrate N-acetyllactosamine, they are expected to produce 2′-FLNAc. This product can also be isolated using a Ulex lectin affinity column. (Fig. 13)

Compositional and structural analyses. All synthetic oligosaccharides, irrespective of their source or mode of synthesis, will be evaluated to confirm their structures by at multiple independent analyses, and to evaluate purity by the most sensitive techniques available.

Microanalysis of sugar ratios by GC (122). The sample is transferred into a capillary tube (1 mm i.d. x 35 mm) in aqueous methanol (50%). The solvent is removed during centrifugation under vacuum. The sample is dried in a vacuum desiccator over P_2O_5. Dry methanolic HCl (0.75 mol/L; 25 mL) and methyl acetate (5 mL) are added before the tops of the tubes are resealed in a flame. The tubes are incubated at 80°C for 2 hand allowed to cool to ambient temperature, whereupon the top of the tube is scored and cracked open. The tube is placed under vacuum with centrifugation to remove the methanolic HCl. Internal standard (methyl heptadecanoate, 2 nmol in 5 ml methanol) is added, and the solvent is removed by vacuum centrifugation. Freshly made 50% acetic anhydride in dry pyridine (5 mL) is added, the tubes are resealed, and the acetylation allowed to proceed for 14 h at ambient temperature (the reaction is complete after 2 h). The top of the tube is scored and cracked open, whereupon an aliquot (1 µL) of the contents are injected into a gas chromatograph fitted with a 30-m DB-1 column. Peaks are detected by flame ionization. After injection into the GC, the temperature is held at 150°C for 15 min and then raised by 4°C per min to a maximum temperature of 300°C. Peak areas are calculated with an HP integrator. This method gives results that are suitable for determining both sugar ratios of a pure compound and absolute quantitation of sugars in a sample. This method yields consistently good results with approximately 1 µg (1 nmol) of oligosaccharide.

Mass spectrometry. All synthetic products will be analyzed by mass spectrometry to assess purity and onfirm structures. The number of components in a sample and their molecular weights are determined by matrix-assisted laser desorption ionization mass spectrometry. MS/MS of peracetylated sample is used to obtain compositional information on the individual components of a mixed sample. The fragmentation pattern in the fast atom bombardment mass spectrum gives some insight into the structure of a pure sample. MS/MS of derivatives can be used to obtain complete structural information even for a sample that contains a major component in the presence of appreciable impurities. Linkage of pure compounds is established by GC/MS analysis of partially O-methylated hexitols and hexosaminitol acetate (PMAAs) (123). Consistent with the program project grant, mass spectrometic analyses will be performed by the Mass Spectrometry Resource Center, Boston University, run by Dr. Catherine Costello.

Polyvalent presentation of the active moiety. In many systems, the polyvalent form of an oligosaccharide ligand can bind with an increased avidity of several orders of magnitude. We will test the affect of such polyvalency by incorporating the active oligosaccharides into neoglycoproteins. As with the synthesis of the oligosaccharides, we will first use syntheses that involve the least development of new technologies, and test the resulting neoglycoproteins to assess whether polyvalency results in an increase in avidity. Then we will develop techniques to synthesize other neoglycoconjugates to determine whether they can result in increased avidity, or further increases, if the first experiments are positive. Finally, we will use the information gained in these studies, and in the synthesis of the oligosaccharides, to try to develop syntheses that are more facile and economical, and that result in products that are most likely to be well tolerated by human infants.

The most conventional approach is to use serum albumin for the synthesis of neoglycoconjugates. Synthetic oligosaccharides are converted into *p*-aminophenyl glycosides, followed by diazotization and conjugation to BSA using standard procedures (124,125). The *p*-aminophenyl glycosides will be prepared ia peracetyl *p*-nitrophenyl glycosides (126-128). The *p*-aminophenyl glycosides (anomeric composition is unimportant for this experiment) will be carefully O-deacetylated (to avoid alkaline hydrolysis) and reduced in the presence of Adams catalyst. The feasibility of this route has been established in preliminary experiments with lactose as a model compound. As an alternative to the unstable diazonium salts, the

stable, usually crystalline isothiocyanates will also be prepared and coupled to BSA as described (124). These compounds will be tested in solid-phase assays to test the affect of polyvalency per se.

For our choice of a protein backbone for neoglycoproteins, we will apply the following criteria: multiple glycosylation sites, low allergenic potential, high perceived acceptability for infant feeding, availability as a high purity food-grade product, and cost. Thus, we want to avoid common allergenic animal proteins like bovine milk casein, egg albumin, and allergenic plant proteins, such as gliadin. Animal blood products are likewise avoided due to perceived risk. However, bovine milk albumin (BMA), the principal whey protein byproduct of the cheese industry, is available in grades widely used by the infant formula companies as a major constituent of their products, and therefore meets our criteria for selection for testing. If this does not prove suitable, other bovine whey products will be tested. Human milk contains polyfucosylated glycoproteins, which will be used as our positive control as we test neoglycoprotein the efficacy of poylfucosylated neoglycoconjugates synthesized from the above sources.

The attachment of the sugars to the protein can be directly without spacers, or through spacers of various chain lengths. As a variety of different chemistries of spacers are available, we will favor the use of spacers whose biodegradation pathways are physiologically common as we test the efficacy of the use of spacers in fucosylated neoglycoconjugates. Of course, the most physiologic of spacers is the core N-linked glycan of a natural glycoprotein. A novel technology has been developed by GlycoFi, Inc., in which yeast has been transfected such as to produce proteins of choice with such glycans attached to their natural glycosylation sites. This company is willing to provide such proteins to us so that we can fucosylate the glycans (by chemical, chemienzymatic, or molecular biological approaches, by substituting this neglycoprotein for lactose as a substrate) to produce a polyfucosylated neoglycoconjugate. These proteins, when used as the substrate for FUT-sc, are expected to produce polyvalent forms of the H-2 epitope. An alternative approach toward the production of these polyvalent H-2 molecules would be to transfect FUT-sc yeast with a plasmid-carrying gene for the human milk protein of choice and the fucosyltransferases needed for the core and lycan structure. Such a construct could produce polyvalent H-2 neoglycoproteins, which could then be .solated by Ulex lectin affinity chromatography. The structures and purity of all neoglycoproteins will be determined by the Mass Spectrometry Resource Center at Boston University described above.

As the research described in this proposal is able to identify the optimum form of the H epitope, i.e., 2′-FL, 2′-FLNAc, or polyvalent H-2 neoglycoproteins, we will focus our efforts toward the most efficient bulk synthesis of the molecule with the greatest avidity, and least allergenic potential.

2) Solid-phase binding

Bacterial strains. Prototype invasive *C. jejuni* strains 166-IP and 287-IP from children with inflammatory diarrhea; *C. jejuni* strain 50-SP, from a healthy child; and two *V. cholerae* strains, El Tor and Classic, will be used for these studies.

Western blot. To assess the ability of campylobacter and *V. cholerae* to bind to histo-blood group antigens, bacterial binding Western blot assays will be performed with DIG-labeled bacteria (23,24). Neoglycoproteins of blood group antigens will be applied to lanes for SDS-PAGE at 6.3×10^{-10} M oligosaccharide per lane. Membranes are washed in TBS, immersed in a DIG-labeled bacterial suspension of 0.2 OD600 and incubated 4 h at room temperature with gentle stirring. Membranes are then washed and incubated for 1 h with the alkaline phosphatase-conjugated anti-DIG antibody, washed and stained with X-Phosphate (5-bromo-4-chloro-3-indolyl phosphate) and Tris-buffered nitroblue tetrazolium in saline (pH 9.5) substrate (Boehringer Mannheim).

3) Cell Binding

CHO cells expressing human receptors for bacteria. α1,2-fucosyltransferase-transfected CHO cells (CHO-*FUT1*), α1,3/4-fucoslytransferase-transfected CHO cells (CHO-*FUT3*), and α1,3-fucosyltransferase-transfected CHO cells (CHO-*FUT4*), and parental CHO cells transfected with the vector pCDM$_7$ lacking the

α1,2 *FUT* gene (CHO-WT) will be used to test bacterial binding and bacterial/host cell agglutination. Parental CHO cells with the vectors will be used as controls.

Bacterial binding to monolayers of transfected CHO cells. The binding of bacteria to CHO cells transfected with the human gene for α1,2-fucosyltransferase (*FUT1*), will be assessed by bacterial-cell association assay. Transfected CHO cells expressing the *FUT1* fucosyltransferase needed for the synthesis of human H-type antigen (α1,2-fucosyl residues) are grown to confluency (28). Controls are wild type CHO cells, parental CHO cells carrying only the plasmid vector, and a clone that expresses the murine UDP-Gal:Galα1,4GlcNAcα1,3-transferase. Monolayers are harvested and seeded into each well of an 8-chamber slide and incubated for 18 h, washed and incubated with a suspension of $9x10^8$ bacteria/mL. Wells are rinsed, fixed with 10% formalin for 1 h, stained by the Warthin-Starry method, and examined under oil immersion with light microscopy, or confocal microscopy for mutant strains with the fluorescent plasmid. Identical preparations grown on round cover slips are examined by scanning electron microscopy after fixing in 2% glutaraldehyde, dehydration through a graded series of solvents, and surface gold deposition.

Inhibition of binding. α1,2-fucosyl ligands and homologs will be tested for their ability to inhibit binding of campylobacter and *V. cholerae* strains to CHO-*FUT1* cells. For molecules that bind to H-2 ligands, including anti-H-2 monoclonal antibodies (anti-H-2 MAbs) and the lectins *Ulex europaeus* (UEA I) and *Lotus tetragonolobus* (Lotus), inhibition is measured on monolayers of CHO-*FUT1* cells incubated in 8-well chamber slides for 1h with each of the α1,2-fucosyl ligands before adding 100 μL of the bacterial suspension containing $1x10^8$ bacteria/mL. For inhibition using homologs to cell surface receptors, including human milk neutral oligosaccharides (Neutral-OS), milk from secretor and non-secretor mothers, neoglycoprotein BSA-H-2 (IsoSep AB, Tullingen, Sweden), and 2′-fucosyllactose, 100 μL of the bacterial suspension are incubated with each of the homologs before being added to the cell monolayer. In both assays, after a 3 h incubation at 37°C, wells are rinsed, lysed with 1% Triton X100, and CFU (colony forming units) of bacteria per well are determined. Data are interpreted as percent inhibition of bacteria association to cells relative to positive controls to which no α1,2-fucosyl ligands or homologs are added.

4) In vivo binding

The effect of neoglycoprotein on campylobacter and *V. cholerae* colonization *in vivo* will be determined in BALB/c mice (weighing 10-20 g). All animal protocols have been reviewed and approved by the Animal Care Committee of the National Institute of Medical Sciences and Nutrition.

Tolerance and safety. Three-week old BALB/c mice will be fed orally either BID (twice daily) or TID (thrice daily) with escalating dose of neoglycoprotein starting at 2 mg/100μL per intake up to 200 mg/μL. Animals will be followed for 2 weeks after the last dose to evaluate for tolerance, weight, presence of diarrhea, and abnormal behavior.

Colonization assays. Two types of experiments are proposed to study the inhibition of campylobacter and cholera colonization *in vivo* using the inbred strain of *mus musculus* Balb/c. (1) *Prophylaxis studies*. In experiments designed to study the ability of neoglycoproteins to inhibit colonization prophylactically, three-week-old female mice are randomly distributed into 2 experimental plus a positive and a negative control group. The potentially therapeutic agents to be tested will be a neoglycoprotein that includes either a 2′-FL or 2′-FLNAc oligosaccharide moiety and a trisaccharide that include either a 2′-FL or 2′-FLNAc oligosaccharide moiety. The two experimental groups are challenged with 10^8 CFU per animal. Two of the four challenge groups are treated with either the neoglycoprotein or trisaccharide 2 days before challenge, on the day of challenge, and 2 hours after challenge, either twice or three times per day. The negative control group will be used to ensure that the animals are initially free from pathogens. The positive control group will receive only saline. Each experimental group will be compared with each other and with the saline control group.
The significance of outcomes will be tested using a chi square test or Fisher's exact test as appropriate. Colonization for the saline group is typically 100%. Setting α=0.05 and two tailed test a sample size of 10

mice per group is adequate to detect an 80% and 60% reduction in colonization in the two challenged groups (i.e., neoglycoprotein or trisaccharide), respectively. Reduction of colonization for the neoglycoprotein and trisaccharide are estimated to be 80% and 60% at the challenge level of 10^8 CFU per animal. Setting α=0.05 and two tailed test a sample size of 91 mice per group is needed. The protective agent neoglycoprotein and trisaccharide will be tested at two dose levels established in the tolerance and safety study. Thus, the number of animals used in the prophylaxis experiment are 91/group x 2 dose groups x2 treatments plus 2 groups of 20 untreated controls (a positive and a negative control group)= 384 animals for campylobacter testing. This number is then doubled to account for cholera testing. (2) *Treatment studies*. In a second series of experiments, neoglycoprotein and trisaccharide will be tested for their ability to clear colonization of animals who are already infected. Again, 3-week-old Balb/c mice are first infected with 10^8 CFU of campylobacter, and after 7 days when the animals exhibit persistent colonization the animals will be treated with the neoglycoprotein or trisaccharide either twice or three times per day at two doses established in the tolerance and safety study. Thus, the number of animals needed are 91/group x 2 dose groups x 2 treatments = 364 animals. This number is doubled to account for cholera testing.

Timeline

Year of Supplemental Project

Activity for supplemental grant	Year 1*	2	3	4
In vitro inhibition by 2′-FL, 2′-FLNAc (Yr 1 PPG)				
Synthesis of oligosaccharide by contractor				
Develop alternative methods for synthesis				
Test sugars produced by alternative synthesis				
In vitro testing of oligosaccharide				
Synthesize large quantity of neoglycoconjugates				
In vivo testing: campylobacter				
In vivo testing: cholera				
Final data analysis and reports				

* Year 1 of proposed supplement is Year 2 of funded Program Project grant (PPG)

E. Human Subjects Research

This proposed study does not meet the definition of human subjects research as defined by the Office of Protection from Research Risks, and therefore 46 CFR Part 46 does not apply.

F. Vertebrate Animals

1a) Tolerance and safety assays. The ability of mice to tolerate various doses of the neoglycoprotein test agent will be tested in three-week-old female mice randomly distributed into 4 experimental groups with a minimum of 10 animals per group and a positive and negative control group. The oligosaccharides will be tested at 2, 10, 20, 100, and 200 mg/μL per intake . Thus, the number of animals used in the tolerance study is (10 animals/group x 5 groups x 1 neoglycoprotein x 2 dose schedules [BID and TID])+ 20 control animals = 220 animals.

1b) Colonization assays. Two types of experiments are proposed to study the inhibition of Campylobacter colonization in vivo using the in-bred strain of *mus musculus* Balb/c. **(1)** *Prophylaxis studies*. In experiments designed to study the ability of milk oligosaccharides to inhibit colonization prophylactically, three-week-old female mice are randomly distributed into 4 experimental groups. Two potentially therapeutic agents will be tested, a neoglycoprotein and a trisaccharide. Two control groups one positive and one negative. Three of the four groups are challenged with 10^8 CFU per animal. The fifth group serves as a negative control to ensure that the animals are initially free from pathogens. Two of the four challenge groups are treated with oligosaccharides 2 days before challenge, on the day of challenge, and 2 hours after challenge, either twice or three times per day. A positive control group will receive only saline. The two potentially therapeutic groups will be compared with each other and with the saline control group, and significance of outcomes will be tested using a Chi-square test or Fisher's exact test as appropriate. Colonization for the saline group is typically 100%. Setting α=0.05 and two tailed test a sample size of 10 mice per group is adequate to detect an 80% and 60% reduction in colonization in the two challenged groups (i.e., neoglycoprotein and trisaccharide, respectively. Reduction of colonization for the neoglycoprotein and trisaccharide is estimated to be 80% and 60% respectively. In order to find a statistically significant difference between the two potential therapeutic agents in terms of reduction in colonization, setting α=0.05 and two tailed test a sample size of 91 mice per group is needed. The protective agent neoglycoprotein and trisaccharide will be tested at two dose levels selected from the tolerance and safety studies. Thus, the number of animals used in the prophylaxis experiment are 91/group x 2 groups x 2 different doses, plus 2 groups of 20 untreated controls (a positive and a negative control group)= 384 animals. **(2)** *Treatment studies*. In a second series of experiments, the neoglycoprotein and trisaccharide will be tested for their ability to clear colonization of animals who are already infected. Again, 3-week-old Balb/c mice are first infected with 10^6 CFU of Campylobacter, and after 7 days when the animals exhibit persistent colonization the animals will be treated with either neoglycoprotein or trisaccharide either twice or three times per day at two doses established in the tolerance and safety studies. Thus, the number of animals needed are 91/group x 2 dose groups x 2 treatments = 364 animals.**(3)** Mouse colonization with *V. cholerae* will be tested using the same experimental design as in the studies indicated above.

1c) Inhibition of *V. cholera* colonization in transfected mice. B6/SJL mice transfected with *FUT* gene in the WAP promoter, which expresses specifically in mammary gland, will be used for these studies. Lactating dams are caged individually with their litters and pups are challenged with 10^2, 10^6, and 10^7 CFU of cholera per animal. To determine colonization, one mouse from each litter (at least 10 individual pups) is sacrificed every 3 days for 10 days (days 1, 4, 7, and 10). The amount of colonization is expressed as percentage of pups positive for Campylobacter at each time period tested. Controls include non-transgenic pups inoculated with 10^6 cholera/animal. The total number of non-transgenic mice used are 10 mice/time point x 5 time points = 50 non-transgenic B6/SJL mice. In addition, total transgenic mice will be 10 animals/time point x 5 time points x 3 inocula = 150 transgenic mice. Thus the total number of mice used for this experiment is 200. This sample size is adequate to detect a reduction of 70% in colonization between the control and any of the inoculation groups on days 7 or 10.

2. The Balb/c mouse is the established model for colonization for Campylobacter and cholera and remains the most convenient mouse strain for its genetic stability and its ability to be colonized. The transgenic B6/SJL is the only transgenic mouse model available in which α1,2-linked fucosyloligosaccharides are expressed in mammary gland during lactation.
3. Animals will be housed and cared for in the animal facility of the Instituto Nacional de Ciencias Medicas y Nutricion. The facility consists of a free-standing building under the direct supervision of Dr. Rafael Hernandez, DVM, and his staff of four additional veterinarians and approximately ten technicians and veterinary students.
4. The inocula of Campylobacter or cholera are provided orally as a liquid suspension in a total volume of 100 μL/animal through a standard feeding tube that does not produce any discernable distress or discomfort in the animals. The same procedure is used for providing the mice with aqueous solutions of oligosaccharides. Mice infected with Campylobacter or cholera do not exhibit symptoms of diarrhea, nor do they show any other discomfort, distress, pain, or injury. Therefore, there is no use of analgesics, anesthetics, tranquilizing drugs, or restraining devices.
5. Mice are euthanized by CO_2 asphixiation at the times indicated in the above protocol. This method is consistent with the recommendations of the Panel on Euthanasia of the American Veterinary Medical Association and the Mexican Veterinary Medical Association.

C. Research Design and Methods

The purpose of the Core is to provide efficient clinical and basic research support necessary to address the specific aims of each project and the program project overall, and to generate a scientific synergy among investigators that makes the program project far more than the sum of its parts. The core will maintain computer databases and specimen banks developed during the previous grant periods; will provide study design, data management and analytic support to project investigators for proposed studies using the databases and specimen bank which now contain more than 60,000 samples from two major cohort studies conducted in Mexico, which are outlined in Figure 4 under Core Aim 1 below; and will conduct several small epidemiologic studies in Cincinnati and Mexico to address specific questions and methodologic issues. The glycobiology core will analyze milk samples collected during the previous and current cohorts for fucosylated and sialylated oligosaccharides. The molecular biology core will genotype and phenotype each participating mother-infant pair in the previous and current cohort studies in terms of their Lewis and secretor histo-blood group types. In general, the core will provide to all projects support for epidemiology and biostatistics, glycobiology, and molecular biology, using a wide range of methods, as needed.

RESEARCH DESIGN

This section of our proposal is organized in relation to the program project unifying themes, which we express as core aims. Under each, we indicate the research design and planned activities of individual cores and the relationship with the specific aims of each project. Following the research design section, we provide a methods section with additional detail regarding aspects of the epidemiology/biostatistics, glycobiology, and molecular biology methods necessary to fulfill these aims.

Core aim 1. Characterize innate and acquired immunologic factors in human milk and the relative contribution of these factors to protection against diarrheal diseases of childhood.

All four projects in this competitive renewal focus on protective oligosaccharide and/or glycoconjugate constituents of human milk, the discovery and characterization of which we have pioneered in this program project. The glycobiology core will measure the neutral and acidic oligosaccharides in milk samples from both cohorts, and the molecular biology core will measure lactadherin in the current cohort. Several of the projects will also examine milk secretory antibody. In addition, the EPEC project will analyze lactoferrin concentration in milk samples from the mothers of all breastfed children in the current cohort. This approach will enable us to simultaneously examine several major mechanisms of human milk protection and the extent to which these mechanisms act independently or synergistically and offer cross-protection against

multiple pathogens. To understand the potential interaction between the innate and adaptive immune systems of human milk, we propose to examine the correlations among these milk variables. The proposed multi-variable studies described below are novel and provide opportunity to achieve important insights into the independence or interaction of major protective mechanisms of human milk.

Current (1998-2003) and previous (1988-1992) cohort studies. Of the 622 mother-infant pairs enrolled in both cohort studies in Mexico, 304 of 306 mother-infant pairs in the current cohort and 286 of 316 mother-infant pairs in the previous cohort breastfed and contributed milk samples for study (total, both cohorts, n=590 breastfeeding mother-infant pairs). The human milk epidemiologic studies under this core aim draw on either the current cohort, the previous cohort, or the combination of the two, depending on the needs of each project, as shown (Figure 4).

The current cohort study conducted in Mexico will be completed by the end of March 2003, with about 3-4 more months needed to complete collection of DNA and saliva samples from study subjects who agree to participate in this protocol. We anticipate that about 70% of breastfeeding mother-infant pairs in the two cohorts will agree to participate in the genotyping/phenotyping protocol (see Core Progress Report). Completion of the fieldwork for this cohort will allow us to finish laboratory analyses of samples and statistical analyses of the data sets. To maximize the power of these cohorts to define oligosaccharide protection against infectious diseases, the glycobiology core will analyze up to 7 milk samples per mother-infant pair in both cohorts, with samples collected at 1-3 weeks, 4-6 weeks, and 3, 6, 12, 18 and 24 months from those who continued breastfeeding. This design will provide a unique and analytically powerful dataset that can definitively characterize human milk concentrations of fucosylated and sialylated oligosaccharides by stage of lactation and allow us to examine milk oligosaccharides as time-varying factors in relation to protection against diarrhea. Given a median duration of breastfeeding follow-up time of 6 months in the previous cohort and 12 months in the current cohort, we anticipate approximately 2000 samples will be analyzed by Dr. Newburg's laboratory using a recently acquired HPLC/Mass Spectrometer to measure both fucosylated and sialylated oligo-saccharides over the years of the proposed grant. In year 1 of the grant, all milk samples collected in the first month postpartum will be analyzed. The remaining milk samples will be analyzed over the course of the grant period.

Number of Mother-Infant Pairs

Subjects enrolled and subgroup inclusion criteria	**Previous Cohort**		**Current Cohort**		**Total**
Total enrolled	**316**	+	**306**	=	**622**
	↓		↓		↓
Breastfed with milk samples	**286**	+	**304**	=	**590**
	↓60%		↓80%		↓~70%
Estimated participants in genotyping/phenotyping protocol	172	+	243	=	**415**

Figure 4. *Subject enrollment flowchart for the previous (1988-91) and current (1998-2003) cohort studies in Mexico. The projects use different subgroups depending on their aims. Bolded are the subject numbers often used to fulfill project aims, as described below.*

In addition, in year 4, Dr. Cleary will analyze lactoferrin concentrations in milk samples collected between weeks 4-6 postpartum from each study mother in the current cohort. Further, in several projects, we will examine the association between pathogen-specific sIgA and the oligosaccharide concentrations in human milk. This comparison of the constituents of the innate and adaptive immunologic systems of human milk allows us to examine potential interactions that could occur between these systems. For example, as demonstrated by Dr. Jiang et al (see Viral Gastroenteritis Project), binding of several major strains of human caliciviruses to saliva is dependent on the individual's histo-blood group type (eg, secretor/non-secretor, and ABO types). We also found that for some calicivirus strains, individual expression of blood group antigens in saliva was associated with the presence of anti- calicivirus antibody in saliva. Thus, it is possible that in

human milk, levels of specific oligosaccharides and secretory antibody are correlated for some pathogens, a hypothesis that we will examine in relation to protection against human caliciviruses, campylobacter and EPEC. This hypothesis is based on the premise that the same maternal genotype that controls expression of ligands involved in pathogen binding in the gastrointestinal tract would also control (a) expression of homologous protective oligosaccharides in her milk and (b) her risk of infection, which would be associated with expression of pathogen-specific sIgA in her milk.

The project-specific studies of maternal milk phenotype and their relation to protection against specific causes of diarrheal disease in children are indicated below by project.

Campylobacter Project. Specific aim 3 of the campylobacter project will use the current cohort (n=306) to determine the protective role of 2'-fucosyllactose (2'-FL) and related compounds in human milk against specific strains of Campylobacter and other causes of bacterial diarrhea. The current cohort will be used because in this cohort, the laboratory in Mexico is typing the strains of Campylobacter. Thus, we can restrict analysis to invasive strains of Campylobacter, which includes approximately 70% of the episodes of Campylobacter diarrhea in children (57). In the current cohort, the incidence of Campylobacter diarrhea is 0.26 cases per child-year of breastfeeding. In a recently submitted manuscript, we demonstrated human milk 2'-FL protection against moderate-to-severe diarrhea of all causes and Campylobacter diarrhea specifically, among the 93 breastfeeding mother-infant pairs in the previous cohort whose samples were analyzed. We will extend this research in the proposed grant cycle. Assuming an incidence rate of 0.18 for invasive strains of Campylobacter infections/child-year, the available sample size (n=306) will provide >80% power, given $\forall$=0.05 and a 2-tailed test, to detect a 2-fold protection by high vs low levels of 2-linked fucosylated oligosaccharide and in particular 2'-FL (H-2) concentration or percent of total oligosaccharides in maternal milk. Further, we can examine 2'-FL in maternal milk as a time-varying factor in relation to protection against symptomatic, strain-specific Campylobacter infection. This project will also examine the association between the maternal milk 2'-FL phenotype and the concentration of anti-campylobacter antibody in human milk.

EPEC Project. Specific aim 5 of the EPEC project will use the current cohort to examine protection by milk antibody, oligosaccharide and lactoferrin on protection against symptomatic EPEC infection. Only the current cohort will be used, because laboratory methods for detection of EPEC were well developed and enacted in this grant cycle. We will begin with a nested case-control study in breastfed infants of anti-EspA milk sIgA protection using milk samples collected just prior to infection. In the current cohort, the incidence of symptomatic EPEC infections is 0.15 episodes per child-year in breastfed infants. Based on this detection rate, we estimate having at least 37 symptomatic first EPEC infections in breastfed children by the end of this cohort, of which 30 (80%) are free of co-infection with any other pathogens. We also anticipate at least 60 asymptomatic EPEC infections. This sample size provides >90% power to detect expected differences in maternal milk anti-EspA antibody in children with symptomatic (n=30) vs. asymptomatic (n=60) first EPEC infections, and is sufficient (>80% power) to support four secondary comparisons of maternal milk antibody protection against symptomatic infection.
After completion of the nested case-control study, we will conduct a longitudinal study to examine the interrelationships and protective effects of all three milk factors - anti-EspA sIgA, oligosaccharide and lactoferrin (individually and combined) - to protect against EPEC diarrhea in breastfed children. All three factors will be assayed in a milk sample collected at 4-6 weeks postpartum. Analysis of the entire cohort will provide >80% power to detect a 2.5-fold difference in the incidence of symptomatic EPEC infection between high and low levels of protective milk factors - sIgA, lactoferrin and oligosaccharide concentration – allowing for a potential correlation of 0.4 among these factors. We will analyze the longitudinal data using Poisson regression to investigate the relative contribution of each of these factors to protect against symptomatic EPEC infection and to control for differing follow-up times per subject. Methodologic justification for conducting both the nested case-control and longitudinal studies is detailed under the Methods section, Epidemiology and Biostatistics Core.

ST Project. Specific aim 4 of this project investigates the association between concentrations in milk oligosaccharides and ST diarrhea in breast-fed infants. This aim will be addressed using the previous cohort

(n=286), from which 7 symptomatic ST infections were detected. Only the previous cohort is used because of laboratory limitations in the measurement of stable toxin in the current cohort. We will measure the concentration of oligosaccharides in milk samples from mothers at weeks 4-6 of lactation, including LNF I and possibly, TF*i*LNO, which may be measured using the new HPLC/MS of the glycobiology core. Milk values will be expressed as concentrations per mL of milk and as a percentage of the total oligosaccharides in the sample; the average concentrations will be compared between the milk of mothers whose children were ST-infected symptomatically (n=7) to those not ST-infected (n=279) while breastfed. Using the concentration of LNF I (H-1) as a surrogate measure of TF*i*LNO concentration, we anticipate that the total sample size of 286 subjects will provide 80% power to detect at least a 1.7-fold difference between the two study groups (symptomatic ST vs not ST-infected) in the mean value for LNF I as a percentage of total oligosaccharides, assuming that the percentage LNF-1 value for mothers whose children were not infected is 0.25. T-tests will be used to compare the mean concentrations of LNF I between the groups.

Viral Gastroenteritis Project. Specific aims 2 and 3 of this project propose to examine the protective effects of specific human milk factors against specific causes of viral gastroenteritis. Aim 2 of this project proposes to characterize factors in human milk that block calicivirus binding to histo-blood group antigens and examine the association between concentration of such factors in maternal milk and prevention of symptomatic infection with human caliciviruses. We will use the combination of the current and previous cohorts (n=590) to examine milk oligosaccharide protection against strain-specific symptomatic HuCV infection. To this end we will investigate the association between increased levels of specific 2-linked fucosylated oligosaccharides in human milk (H-1, H-2, Le^b and Le^y) and the decreased risk of symptomatic infection with specific strains of human caliciviruses that are secretor binding (i.e., 387, NV, and MOH strains). Together, these three strains are estimated to account for more than 80% of all human calicivirus infections in our study population, with the 387 strain alone accounting for about 70% of all symptomatic calicivirus infections. The incidence of all strains of human calicivirus infection in the current cohort is 0.39 symptomatic infections per child-year. Assuming an incidence rate of 0.23 for symptomatic, 387-associated calicivirus infections/child-year, the available sample size (n=590) will provide >80% power, given $\forall$=0.05 and a 2-tailed test, to detect 1.6-fold protection by high vs low levels of 2-linked fucosylated oligosaccharide in maternal milk. This sample size also will provide approximately 80% power to detect 3.5-fold protection against symptomatic infection with NV and MOH strains of calicivirus by high vs. low levels of 2-linked fucosylated oligosaccharides in maternal milk, assuming incidence rates of 0.04 cases per child-year with each of these pathogens.

Further, as part of its activities under project Aim 3, the Viral Gastroenteritis project will assay lactadherin in the milk samples of all mothers in the new cohort. Our previous analysis of lactadherin levels in maternal milk samples (see Core Progress Report above) indicates that lactadherin decreases modestly but systematically over the first four months of lactation. There is no evidence that lactadherin levels change in response to maternal exposures. Thus, we plan to analyze lactadherin concentrations in a single milk sample from all study mothers collected at a standardized time of lactation, ie, the first month postpartum. This sample will be analyzed in relation to protection against symptomatic infections with rotavirus infection and human caliciviruses to test the hypothesis that this is a cell adhesion molecule with broadly anti-viral properties. Our previously published manuscript on human milk lactadherin demonstrated, with only 31 subjects, 12-fold protection against symptomatic RV infection in breastfed children consuming milk with high lactadherin levels (above the mean). The sample size of the current cohort provides >80% power to detect a 2-fold protection against infections from rotavirus and all strains of human caliciviruses by low versus high levels of lactadherin in maternal milk assuming an incidence rate of 0.3 /child yr for rotavirus (61) and .39/child-yr for calicivirus.

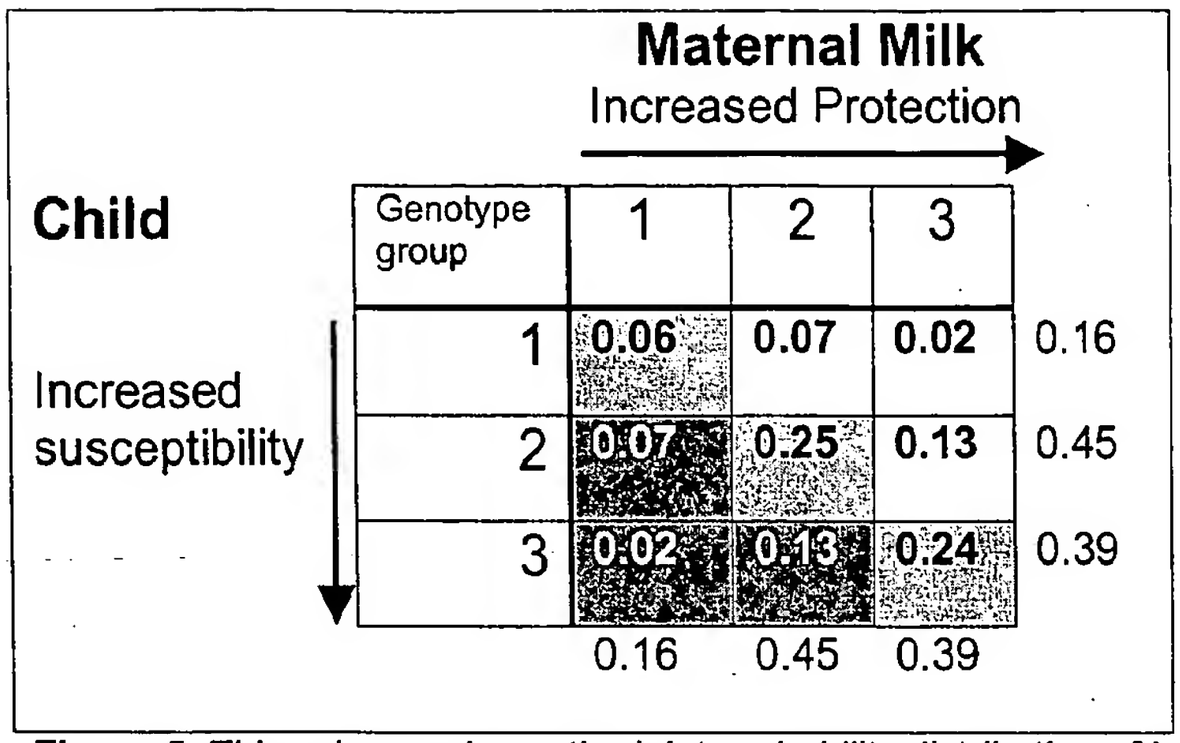
Comparative longitudinal studies of the expression of milk oligosaccharides in Mexico and US populations. For comparison with the Mexican study cohorts, in which we propose to analyze serial milk samples collected up to 2 years postpartum, we plan to conduct a small longitudinal study of human milk oligosaccharide expression in Cincinnati. To examine the longitudinal profile of human milk expression over the first 2 years postpartum, we will enroll 30 volunteer mothers, with milk samples collected every week in the first month postpartum, and monthly thereafter. Inclusion criteria will include intention to breastfeed to

two years postpartum and willingness to allow analysis of the DNA of milk cells for testing Lewis and secretor genotypes. Milk samples will be collected in a standardized fashion as consistent with the studies conducted in Mexico. We will provide study mothers with portable Medela breast pumps, and will offer lactation counseling support as requested. Prior to starting the study, we will explore strategies for enrolling mothers who intend (and are likely) to breastfeed for 18-24 months (eg, La Leche League volunteers). The longitudinal expression of milk oligosaccharide will be compared between Cincinnati and Mexican study populations, and most importantly, by maternal genotype, which we expect to affect the longitudinal pattern of milk oligosaccharide expression. This Cincinnati volunteer study will be overseen by Dr. Mary Staat with support from a study nurse, Dr. Morrow and staff. The milk samples collected will be sent to the laboratory of Dr. Newburg for analysis. We propose to begin enrollment mid-year 1. We estimate an average of 1-2 women enrolled per month, completing enrollment within 2 years. Study followup will continue up to year 5 of the grant cycle.

Core Aim 2. Determine the genetic basis for variability in human milk oligosaccharide protective factors and in infant susceptibility to diarrheal diseases

During this grant cycle the molecular biology core will characterize the secretor and Lewis genotypes of the mother-child pairs involved in the current and previous cohort studies (see also Molecular Biology Core section below), in collaboration with the Mexico core, and will analyze the expression of phenotype in saliva samples collected from study children. This work will be undertaken to further our understanding of human histo-blood group antigens as receptors for several enteric pathogens (see Campylobacter, ST-associated *E. coli* and Viral Gastroenteritis projects of the grant), and to test our understanding that variable expression of milk oligosaccharides is the result of polymorphisms in the genes that control histo-blood group type. Thus, genotyping and phenotyping both mother and infant is necessary to clarify maternal milk protection, infant susceptibility, and their interrelationship. As noted in the progress report of the Core, we anticipate that 415 (70%) of the 590 eligible mothers and children from the previous and current cohorts will participate in the collection of saliva samples necessary to fulfill this core aim.

Once the secretor and Lewis genotypes and phenotypes of individual mothers and their children are characterized, we can examine the relationship between maternal genotype and milk phenotype and the relationship between infant genotype and saliva phenotype. Maternal secretor and Lewis phenotypes and infant secretor and Lewis phenotypes will then be examined in relation to infant risk of infection. For at least several pathogens, risk of symptomatic infection in children is likely to be associated with the degree of phenotypic expression of 2-linked structures in the gastrointestinal tract of the child and in the mother's milk. Maternal milk genotype and infant saliva genotypes will be examined one gene at a time (eg, secretor genotype only [sese, Sese, SeSe], and Lewis genotype only [lele, Lele, LeLe]). In addition, consistent with an increasing literature, we will classify individuals' risk status based on the joint distribution of secretor and Lewis genes, which provide nine or more unique combinations, depending on the number of gene mutations identified. As shown in the oligosaccharide synthesis pathway shown in the ST project, the fucosyltransferases encoded by the Lewis and secretor genes appear to compete and thus affect the degree to which specific oligosaccharide structures are synthesized. In a study of genetic polymorphisms in Lewis and secretor genes and risk of infection with *Helicobacter pylori* infection, Ikehara et al (42) reported that type 1 Lewis antigen expression was dependent on the combination of both genotypes, and that *H. pylori* infection rate was positively associated with the number of Se alleles and negatively associated with the number of Le alleles. When subjects were classified by the combination of both genes, the odds of infection was 10-fold greater in the genetically high risk group (the SeSe/lele, SeSe/Lele, Sese/lele) compared to the lowest risk group (sese/LeLe).



Expected infant risk of infectious diarrhea (population frequency)	
High (0.22)	High susceptibility/low protection
Middle (0.55)	Susceptibility=protection
Low (0.22)	Low susceptibility/high protection

***Figure 5**: This schema shows the joint probability distribution of Lewis and secretor genotypes for the mother-infant pairs in our Mexican study population. Lewis and secretor genotype combinations are classified into three groups, as described in the text above. Each column and row refers to groups 1 to 3: 1 - all non-secretors (sese); 2 - all Se genotypes in which the number of Se alleles≤the number of Le alleles; 3 - all genotypes in which the number of Se alleles>the number of Le alleles. For the same genotype group, the child's status indicates degree of susceptibility and the mother's status indicates degree of protection offered by her milk. We hypothesize that the breastfed infant's risk of infection is dependent on the combination of mother and child's genotypes and the extent to which they are concordant (est 55% of the population in which susceptibility=protection, the diagonal) or discordant. If their status is discordant, some infants who have low susceptibility to infection may receive a highly protective milk (22%) and thus may be especially protected, while some infants who are highly susceptible may receive little protective substance in their milk (22%) and thus may be especially at risk of symptomatic infection.*

Our analysis of the relationship between maternal and infant phenotypes and genotypes will begin with thorough description of the phenotypic profiles associated with each identified unique individual genetic combination. For purposes of hypothesis testing, we also propose to use a joint secretor and Lewis genotype risk classification similar to that of Ikehara et al (42). Group 1 will consist of all nonsecretors or low secretors, i.e, all sese (anticipated to be approximately 16% of the Mexican study population, including the 2% who express no 2- linked fucosylated oligosaccharides and an additional anticipated 14% who may be "partial secretors," expressing little 2- linked fucosylated oliogosaccharides). Group 2 will consist of individuals whose secretor and Lewis genotypes are expected to produce an intermediate level of 2-linked structures. We anticipate this category to be defined by individuals in whom the number of active secretor alleles (Se) is less than or equal to the number of active Lewis alleles (Le): (Sese/Lele, Sese/LeLe, SeSe/LeLe). We estimate that this may be approximately 45% of the Mexican study population. Group 3 will consist of individuals whose secretor and Lewis genotypes are expected to produce the maximum quantity of 2-linked oligosaccharide structures. We anticipate that this group is defined by individuals in whom the number of active secretor alleles is greater than the number of active Lewis alleles (Sese/lele, SeSe/lele, SeSe/Lele), approximately 39% of the study population.

For each genotype group, we hypothesize that the mother's genotype defines the degree of protection offered by her specific milk oligosaccharides while the same genotype in the infant indicates degree of risk for infection. Data analyses will be conducted to test this hypothesis. We will apply this classification to mothers and children separately, based on their individual genotypes, with final analyses accounting for the joint classification of the status of the mother and child. Figure 5, shown above, provides a concept model for understanding the mother and child joint genetic protective and risk status. While we plan to apply the joint classification concept shown in figure 5 to our summary data analyses, other approaches will also be applied to data analyses that include both mother and child (see Methods, Epidemiology/Biostatistics Core below). Finally, for each mother-child genotype and phenotype combination we will compare infant risk of infection for the postbreastfeeding vs. breastfeeding periods. We hypothesize that high-risk infants who are well protected while breastfeeding may have the highest postbreastfeeding risk.

In summary, as a result of innate variation in concentration of protective oligosaccharides in human milk, mothers may offer their breastfed infants differing levels of protection. Similarly, as a result of innate variation in expression of histo-blood group antigen glycoconjugates on host cell surfaces, breastfed infants may differ in their risk of infectious diseases. To date, our studies and others' have been based on phenotypic analyses only, including characterization of the histo-blood group antigens and/or their homologs present in milk and saliva. As a result, this core aim potentially represents a novel and significant contribution to advancing research on human milk and child health.

The application of the genotyping and phenotyping work of the core to the previous and current cohort studies conducted in Mexico is described below for each project. Further detail on analytic methods is provided in the Core Methods section following.

Campylobacter Project. Specific aim 3 of this project is to determine the genetic basis for variability in secretor phenotypes expressed in maternal milk and infant secretions, and the genetic basis for infant susceptibility to Campylobacter and other bacterial diarrheal diseases. Specifically, we will describe, among the nine distinct secretor and Lewis histo-blood group genotypes, the pattern in the mean maternal milk concentrations of 2-linked fucosylated oligosaccharides, particularly 2'-FL (H-2). For this specific aim we will utilize both current and previous cohorts (N=415). For hypothesis testing purposes, we will initially classify the distinct genotypes into 3 genetic groups based on our current understanding of the potential dominance of of the secretor gene in relation to the Lewis gene (described above). Using the mean concentration of 2'-FL from a previous study, the available sample size of 415, α=0.05 and 2-tailed test, will provide > 90% power to detect significant differences among these three genotype groups, and >80% power to detect significant differences between any 2 groups. We also want to investigate the risk of campylobacter infection among the genotype groups based on the infant saliva. Assuming that the incidence of invasive strains of Campylobacter infection by genetic risk groups is: 0.11 for group 1 (non- and low secretors), 0.18 for group 2 (medium secretors) and 0.29 for group 3 (high secretors), we will have >80% power (given $\forall$=0.05 and a 2-tailed test) to detect a significant difference among these groups. This sample size will also provide us with approximately 80% power to detect a 2.6 fold difference in the incidence of invasive strain Campylobacter between the low and high secretor genotype groups.

EPEC Project. The EPEC project proposes to examine susceptiblity to disease associated with histo-blood group type. At this time, we are not ready to hypothesize a specific gene or set of genes that control genetic risk for EPEC disease or milk protection, but over the years of this grant cycle we anticipate identifying a specific histo-blood group type and the probable genetic polymorphismis that control varied expression of risk and milk protection. The DNA samples collected in Mexico would then allow testing for polymorphisms in identified genes.

ST Project. Specific aim 4 of this project proposes to determine the genetic basis of variability in the concentrations of oligosaccharides in human milk that protect against ST. To fulfill this aim, we will examine the expression of oligosaccharides in milk in relation to maternal genotype. Specifically, we will describe, among the distinct secretor and Lewis histo-blood group genotypes, the pattern in (a) the mean maternal milk concentrations of TF*i*LNO or its surrogate, LNF I and (b) the mean value of individual maternal samples of the ratios of milk 2 to 3 linked fucosylated oligosaccharides. For hypothesis testing purposes, we plan to classify the distinct genotypes into 3 groups based on our current understanding of the potential dominance of of the secretor gene in relation to the Lewis gene (described above). Using the mean concentration of LNF I as a surrogate measure for TF*i*LNO, and with the available sample size of 415, α=0.05 and 2-tailed test, we anticipate > 90% power to detect significant differences among these three groups, and >80% power to detect significant differences between any 2 groups. We will use analysis of variance and two-sample t-test for comparison of milk values by genotype groups. Correlation analysis will be used to determine the relationship between the milk concentration of TF*i*LNO and the ratio of 2 to 3 linked fucosylated oligosaccharides.

Viral Gastroenteritis Project. Specific aim 1 of this project proposes to characterize the genetic basis for phenotypic expression of histo-blood group antigens in children that are associated with risk of human calicivirus infection. To achieve this aim we will examine the risk of strain-specific symptomatic calicivirus infection among the three genotype groups (discussed above). In particular, we wish to investigate if there is a linear trend in the risk among these groups in the following increasing order: non and low secretors (Group 1), medium secretors (Group 2), and high secretors (Group 3). Assuming that the incidence of strain-specific symptomatic calicivirus infection for these groups is 0.12, 0.19 and 0.30, respectively, we will have >80% power (given ∀=0.05 and a 2-tailed test) to detect a significant difference among these groups. This sample size will also have 80% power to detect a 2.5 fold calicivirus incidence between the low and high secretor groups. We also will investigate the risk of calicivirus infection based on the ABO blood group type. Specifically, we will compare risk status between the A blood group (about 20% of the Mexican population) and the O blood group (about 67% of the Mexican population). Thus, in our cohort of 415 participants, we anticipate 83 and 278 subjects with blood groups A and O respectively. Assuming that the incidence rate for calicivirus infections in blood group A is 0.38 cases/child-yr, given ∀=0.05 and a 2-tailed test, this sample size provides at least 80% power to detect a two fold increased risk of infection in the A blood group compared to the O blood group. Further, we also wish to investigate the protective association between the 2-linked fucosylated oligosaccharides in maternal milk dependent on the concordance of the mother and child's ABO blood group types. We will examine the hypothesis that the association between milk oligosaccharide and protection of the breastfed child depends on the match between the mother and the child's blood group. Assuming that the risk for symptomatic calicivirus infection in the concordant group is 0.2 cases/child-yr, this sample size (given ∀=0.05 and a 2-tailed test) will provide >80% power to detect at least a 1.8-fold increased risk of calicivirus infection in the discordant compared to the conconrdant mother-infant pairs. Data analysis will be conducted using chi-square or Fisher's exact test where appropriate. The relationship between protective factor and risk of calicivirus infection will be investigated using a generalized linear model with a Poisson link function.

Core aim 3. Characterize pathogen binding to cell surface receptors and the inhibition of pathogen-cell surface binding by human milk factors.

Each of the projects will characterize the cell-surface receptors and their relationship to pathogen binding, and binding inhibition by human milk factors. Description of these aims and methods are found in each project. The following are two small studies to be conducted in Cincinnati and Mexico which are designed address this Core aim using a molecular epidemiologic approach.

Ontogeny of expression of histo-blood group antigens in infants. In order to characterize infant susceptibility to pathogen binding by developmental stage, we propose to conduct a small longitudinal study of the changes in expression of histo-blood group epitopes in the saliva of non-breastfed infants from birth to 2 years postpartum. This methodologic study is being conducted by the core investigators to support the work of the projects and the program project as a whole. We propose to enroll *non-breastfed* infants in Mexico (n=20) and in Cincinnati (n=20) from birth to 24 months, and collect saliva (for Lewis antigen) and stool (to determine colonization or infection status) in a standardized manner at 1-2 weeks, 3-4 weeks, and every 2 months thereafter. All samples will be collected in the morning, with the time of day and health status of the donor noted. Since non-secretors comprise approximately 20-25% of the population in Cincinnati, we may find approximately 4 to 5 non-secretors without any effort to screen and identify this group. But given that the longitudinal pattern of Lewis antigen expression is expected to differ between secretors and non-secretors, we will continue enrollment until at least 5 non-secretor infants are included. We will begin enrollment at both sites by the middle of year 1 and will continue with enrollment and follow up through year 4 of the grant cycle.

Surveillance study in Cincinnati. Over the next three years, a hospital surveillance study being conducted by Dr. Mary Staat will characterize the causes of admission for diarrheal disease at Cincinnati Children's Hospital Medical Center. Approximately 250 cases of diarrhea will be studied each year. Drs. Jiang and Morrow will be involved in this study to characterize the association between cause of diarrhea (strain-

specific human calicivirus infection, rotavirus, or other), expression of histo-blood group antigens in saliva, and saliva binding to Norwalk-like viruses (NLV). A minimum volume of 1 mL (optimal volume 10 mL) of saliva will be collected from study children using a special suction device designed for this purpose. The saliva samples collected will be sent to the laboratory within a few hours for processing, or immediately frozen at –20°C. For determination of histo-blood group antigen and saliva binding to Norwalk-like viruses, the saliva samples will be heated and clarified by centrifugation. Stools are being tested in Dr. Jiang's laboratory for the presence of Norwalk-like viruses and rotaviruses using RCT-PCR. Based on preliminary analysis of samples, approximately 5-7% of all hospitalized cases are expected to be due to human calicivirus. Therefore over the next three years, we anticipate about 40 or more cases of human calicivirus infection in study children; most are likely to be associated with the 387 strain. Saliva samples from approximately 40 healthy control children will be obtained from a well-child clinic at CCHMC, matched on age and season of caliciviruses cases. We hypothesize that all of the children with 387-associated calicivirus diarrhea will be secretors, whereas controls will represent the expected population distribution.

Core aim 4. Synthesize oligosaccharide epitopes found in human milk and test their protective efficacy through pre-clinical studies that lead to clinical trials

In this application for renewal, we propose to conduct a set of pre-clinical studies involving oligosaccharide synthesis and extensive animal testing to determine the most effective oligosaccharides for inhibition of specific pathogens. Our proposed animal studies examine the efficacy of various natural and synthetic tri- and tetrasaccharides (eg, 2'-FL, 2'-FLacNAc, and other oligosaccharides). These studies are designed to lead to well-targeted human phase I and phase II clinical trials. Anticipating such trials, the key questions to be addressed by our proposed pre-clinical studies are: 1) the relative efficacy of a panel of similar or related unbound oligosaccharides; 2) the impact of timing of oligosaccharide administration relative to the onset of infection; and 3) the impact of escalating oligosaccharide doses in terms of safety, tolerance and pathogen inhibition. Each of these questions addresses critical dimensions of the design of future trials and the dose administration of oligosaccharides that might be given to children for prophylaxis or treatment purposes. Towards the end of this grant cycle, we will also explore which of the human milk proteins to use as the structural scaffolding to ultimately test multivalent and polyvalent forms of oligosaccharides. The specific pre-clinical studies that address this aim are described under the Campylobacter, ST and Viral Gastroenteritis projects.

Even as we conduct the pre-clinical animal studies supported by this program project grant, we propose to submit applications to NIH through the SBIR mechanism to request funding for the translational research studies that we plan to conduct over the next 5 years. These proposed trials in human subjects will be undertaken by the same team of investigators as a systematic progression from the preclinical research conducted in this program project. In human trials funded by the SBIR mechanism, we will test the most active synthetic candidates that demonstrate protection against campylobacter and ST-associated *E. coli* in the suckling mouse. We also propose to study oligosaccharide or glycoconjugate protection against human caliciviruses guided by *in vitro* studies and clinical evidence of the association between virus binding and subjects' histo-blood group type. The synthetic oligosaccharides and/or glycoconjugates to be used in human studies will be produced in a laboratory that is certified to maintain good manufacturing practices (GMP). The glycobiology core has identified a GMP source of synthetic oligosaccharides. The human studies for which we will request funding under the NIH SBIR mechanism will be: 1) safety and tolerance studies in children in the US and Mexico; and 2) pilot efficacy (Phase II) trials in children. In preparation for these studies, we have initiated contact with Drs. Elizabeth Yetley and David Hattan at the Center for Food Safety and Applied Nutrition, Food and Drug Administration. In addition, we have identified new collaborators whose expertise will be helpful in the development and conduct of human trials, including two senior investigators at Cincinnati, Dr. Mitchell Cohen (pediatric gastroenterology and infectious disease) and Dr. Alexander Vinks (pediatric pharmacology research unit), who have agreed to collaborate with us on the development of our phase I and phase II clinical trials.

METHODS

In this section we describe in greater detail the methods that may be used, and issues that may need to be addressed by each component of the Core.

Epidemiology and Biostatistics Core. In addition to Dr. Morrow, this core includes Dr. Mekibib Altaye (biostatistics); Jareen Meinzen-Derr, MPH, the Project Coordinator, who has substantial experience in data management and data analysis; Dr. Mary Allen Staat, who has significant experience and infrastructure for conducting clinical studies in the Cincinnati population; and Dr. M. Lourdes Guerrero, who heads the field team in Mexico but works closely with Dr. Morrow and investigators at all sites to complete project aims.

Table 5. Examples of the methodologic issues and approaches considered by the Core

Methodological issues	Approaches to the issues
Measurement of "dose exposure" to molecular constituents of milk and human milk per se	+ Standardized timing and method of milk sample collection. + Variability in milk measure using a referent standard or denominator, eg, consider 2' FL as a percentage of total oligosaccharide. +.Address exposure to human milk using international breastfeeding classifications (eg exclusive, predominant,partial) and/or calculate breastfeedng as percentage of total feedings. + Develop a population percentile reference to rank individual values relative to population
The time-varying nature of human milk factors, including their diverse patterns and correlations over lactation, and the parallel developmental changes in infants	+ Document correlations and longitudinal patterns and incorporate several milk measurements as appropriate. + Conduct methodologic comparison of "prior to infection" vs early milk sample values in relation to infection outcomes + Use models that allow for complex correlated data as needed
Correlation between independent variables, including potential multicollinearity	+Investigate if the correlation between independent variables over time is modest or a serious limitation in relation to a potential causal model. + If correlation is major consider analysis of one factor at a time and/or development of a combined variable (eg, indicator)
Potentially complex interaction in the data, eg, if pathogen specific protection by milk oligosaccharide is dependent on the concordance of mother/infant pair blood groups	+ Anticipate and look for interaction Use appropriate subgroups and analyze the data accordingly + Use kappa-like statistics to analyze the data
Statistical power to address analytic needs, eg, multiple comparisons	+ The power requirement for each specific project is addressed in advance + "Fishing" expeditions are avoided by pre-specification of hypotheses that are driven by in vitro/in vivo data When lack of power is indicated the nature of the analysis will be qualitative or descriptive.

The epidemiology/biostatistics group will be responsible for the design and analysis of a number of clinical and animal studies in this proposed renewal as described above and in the project sections. A variety of statistical methods will be used to address project aims. These descriptive statistics and methods used for simple comparisons such as chi-square, Fisher's exact test, t-tests, correlations, analysis of variance, and kappa-like statistics for studies of concordance. For the longitudinal and more comprehensive analyses we will use modeling approaches, including generalized linear models with logit or Poisson link functions to address within and between subject correlations, and time to event models that address repeat infections. In the design and analysis of our studies, we are aware of methodologic issues that require special consideration, as summarized in Table 5 above. All of these approaches are well within our experience and

in some cases, are methodlogic contributions that we have published in the current and previous grant cycles (18, 64-67). Our computer hardware capacity is more than sufficient to manage and analyze the research datasets of the program project (see Resources page). We have access to a variety of statistical software, including SAS, STATA, and S-plus, that we will utilize as appropriate to conduct data analyses.

The longitudinal and comprehensive nature of our data will allow us to conduct novel and powerful analyses of milk factors never previously possible, eg, of the distribution of milk oligosaccharides over two years of lactation, and of the interrelationships between multiple protective factors in milk – lactoferrin, lactadherin, fucosylated and sialylated oligosaccharides and secretory antibody, which could establish an invaluable population reference for human milk immunologic factors.

Glycobiology Core. This core continues to be led by Dr. David Newburg and team at the Shriver Center, Program in Glycobiology, University of Massachusetts Medical School. The glycobiology core will provide expert support to each of the projects by isolating human milk oligosaccharides, characterizing biologically active oligosaccharides, and synthesizing oligosaccharides, glycoconjugates, and other biochemicals. The Core also undertakes methodologic studies as needed and works with the Epidemiology core to determine the amounts and kinds of specific oligosaccharides in milk samples from different mothers. The methods needed from this core over the next few years to support specific project aims are described below.

Qualitative and quantitative measurement of oligosaccharides in milk from different mothers. (Campylobacter Project, Aim 3; Stable Toxin Project, Aim 4; Viral Gastroenteritis Project, Aims 1, 4). We developed and utilize a sensitive method for routine identification and quantification of intact neutral (fucosylated) human milk oligosaccharides. The isolated neutral oligosaccharide fractions are perbenzoylated, resolved by reversed-phase HPLC, and detected at 229 nm. This method resolves most structural isomers and does not require stringent removal of lactose. Peaks are detected at the low nanogram (pmol) level, and peak areas are linear from 1 to 1,000 µg for a standard oligosaccharide. Oligosaccharide samples equivalent to those found in one microliter of milk yield optimum chromatographic separation and resolution. The method has a mean coefficient of variation of 13%. The major peaks in human milk co-elute with authentic oligosaccharide standards ranging from tri- to octasaccharides, and their identities are confirmed by mass spectrometry. High-performance capillary electrophoresis of un-derivatized acidic oligosaccharides, with detection by UV absorbance at 205 nm, has proven sensitive to the femtomole level in studies of variation in acidic milk oligosaccharides. Eleven standard oligosaccharides ranging from tri- to nonasaccharide (3'-sialyllactose, 6'-sialyllactose, 3'-sialyllactosamine, 6'-sialyllactosamine, disialyltetraose, 3'-sialyl-3-fucosyllactose, sialyllacto-*N*-tetraose-a, sialyllacto-*N*-tetraose-b, sialyllacto-*N*-neotetraose-c, disialyllacto-*N*-tetraose, and disialomonofucosyllacto-N-neohexaose) have been resolved; baseline resolutions of 3'-sialyllactose, 6'-sialyllactose, and other structural isomers have been achieved. Peak areas were linear from 30 to 2,000 pg and were reproducible with a coefficient of variation between 4% and 9%. There was no evidence of quantitative interference of one oligosaccharide with another. This sensitive, highly reproducible method requires only a basic sample work-up.

Isolation of human milk oligosaccharides and separation into individual components. (EPEC Project, Aims 1, 4 and 5; Viral Gastroenteritis Project, Aims 2 and 4). Previously frozen pooled human milk (1 liter) is centrifuged at 4˚C at 3000 x *g* for 1 h. The cream is removed and the skimmed milk filtered through glass wool. The filtrate is mixed with equal parts of ice-cold acetone to precipitate macromolecules and stirred overnight at 4˚C; the precipitate is removed by centrifugation at 3000 x *g* for 45 min. The skimmed deproteinated milk fraction is reduced to ~200 mL by rotary evaporation and the resultant aqueous solution applied to a 1-liter charcoal-Celite column (72) followed by several rinses with distilled water. Ten liters of 5% ethanol in water is run through the column to remove lactose. The total oligosaccharide fraction is then eluted from the column with 65% ethanol. The total oligosaccharide fraction is separated by application of 5 mg to a 50-mL *Ulex europaeus* lectin column (Vector Laboratories, Burlingame, CA) and washed with pH 7.4 bicarbonate buffer (15 mM $NaHCO_3$, 150 mM NaCl, 0.1 mM $CaCl_2$, 6 mM NaN_3), yielding 4.6 mg of non-lectin binding oligosaccharides. The retained fucosylated oligosaccharide fraction is then eluted from the lectin column with a high-salt buffer (15 mM $NaHCO_3$, 1M NaCl, 0.1 mM $CaCl_2$, 6 mM NaN_3), pH 7.4, yielding 0.5 mg. Both fractions are isolated from their buffers by passage through a charcoal-Celite (1:1,

wt:wt) column, washing with 5% ethanol, and elution with 65% ethanol. The fucosylated oligosaccharide fraction is resolved by semipreparative HPLC on a 250 x 4.6 mm column containing 5-μ amino bonded silica (Rainin). The samples are applied to the column on acetonitrile/water (85:15) and are eluted by a 90-min linear gradient of mobile phase at 1 mL/min that terminates at acetonitrile/water (30:70). Peaks were detected at 195 nm. Selected subfractions were further resolved by reversed-phase HPLC chromatography using an Alltech 150 x 4.6 mm 3 μ C18-silica column with water as the mobile phase (0.5 mL/min for 15 min) and absorption at 195 nm. *N*-linked carbohydrates are released from a sample of purified lactadherin or glycoprotein by reaction with *N*-glycanase (peptide: *N*-glycosidase F; Genzyme Inc) employing previously described methods (73). The active fraction (2 mg/mL) undergoes reaction with 15 mU of enzyme dissolved in 0.2M sodium phosphate, pH 8.6, at 37°C for 18 hr. The material is then dialyzed, lyophilized, and analyzed for activity.

Structural analysis of oligosaccharides isolated from human milk or human milk glycoconjugates (Viral Gastroenteritis Project, Aim 4). Isolated carbohydrate structures will be determined by a combination of classical chemical techniques, sequential enzymatic degradation, and state-of-the-art mass spectrometric (MS) analysis in conjunction with nuclear magnetic resonance (NMR) analysis. A microscale analysis of the sugars of oligosaccharides by gas chromatography has been added to our Glycobiology Core procedures. The major modification is that sample size is reduced, and reactions are run in a sealed capillary tube under an inert atmosphere. This method yields results that are suitable both for determining the molar sugar ratios of a pure compound and for absolute quantitation of sugars in a sample. Oligosaccharides are permethylated according to the solid NaOH in the dimethylsulfoxide and iodomethane procedure described by Larson et al. (74) and Gunnarsson (75), and the permethylated samples are purified by reversed-phase chromatography. Linkage is established by GC/MS analysis of partially O-methylated hexitols and hexosaminitol acetates (PMAAs). Quantitation of PMAAs is by selected ion chemical ionization peak areas. Deduced linkages will be confirmed by electrospray MS/MS of the permethylated compounds, a technique that has been successfully used to confirm the structures of milk oligosaccharide HPLC peaks. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF) is now the method of choice for molecular weight determination of any unknown molecule, including oligosaccharides.

Synthesis of combinatorial libraries. (Campylobacter Project, Aim 4; Stable Toxin Project, Aim 1; Viral Gastroenteritis Project, Aim 3). We have devised two approaches for the synthesis of a combinatorial library of unbranched fucosyloligosaccharides that encompass the possible products of the Lewis and secretor gene family. The first utilizes a scheme of automated synthesis. Our second approach, employing conventional batch synthesis using well-established techniques, will be used when automated synthesis is not feasible. The details of both of these synthetic schemes are described in the Experimental/Research Design and Methods section of the Stable Toxin Project.

Molecular Biology Core. In this competitive renewal, the molecular core will continue to assist individual subprojects to fulfill their specific aims. This core will continue to be led by Dr. Xi Jiang. Dr. Tibor Farkas, who began as a research fellow in Dr. Jiang's laboratory, is now a research instructor and a co-investigator in the core. Dr. Jacques Le Pendu, a glycobiologist and human histo-blood group antigen expert, will serve as a consultant to the core. Dr. LePendu has collaborated with Dr. Jiang in the past 2 years on the Norwalk Virus (NV) histo-blood group antigen studies (38, see also Viral Gastroenteritis Project). The interactions of the molecular core with each project is described in the research plan of individual projects but briefly summarized here. The core will prepare baculovirus-expressed site-directed mutagenesis modified lactoferrins for aim 3 of the EPEC grant.

This core will be responsible for conducting genotyping of mother-infant pairs in the cohorts in collaboration with Dr. Ruiz-Palacios and Dr. LePendu. Histo-blood group antigens are glycoconjugates (complex carbohydrates linked to lipids, proteins, carbohydrates, and/or other moieties) present on the surface of red blood cells and mucosal epithelial cells, or as soluble, unbound antigens in biological fluids such as blood, saliva, intestinal contents, and milk. These antigens are synthesized by sequential additions of monosaccharides to the antigen precursors by glycosyltransferases encoded by genes that control ABO and Lewis blood group types: ABH (*FUT1*), secretor (*FUT2*), and Lewis (*FUT3*) genes. The existing

scientific literature regarding histo-blood group antigen systems has been largely derived from studies in populations of European descent. There is limited information on the genetic polymorphism of histo-blood group type in the Mexican population. There is also a lack of information on histo-blood group antigen expression in the early life of the newborn, which may play a critical role in the infant's susceptibility or resistance to many infectious pathogens. Finally, oligosaccharides with similar structures or epitopes of human histo-blood group antigens have been identified in human milk which we have shown to be associated with protection of breastfed infants from diarrheal diseases in our cohort study in Mexico (see Core Progress Report).

Because published data are not available regarding the phenotypic expression of histo-blood group antigens in the Mexican population, we will first phenotype our study cohort participants. The histo-blood group genes of phenotypically negative individuals as well as some positive controls will be analyzed. We will focus on the three major gene families (*FUT1, FUT2*, and *FUT3*), which encode different fucosyltransferases that determine the H, secretor and Lewis phenotypes. In European populations, the frequency of the inactive *FUT2* gene is about 20% and the frequency of the inactive *FUT3* gene is about 10%. Preliminary results suggest that in Mexico there is a lower prevalence of individuals (2-4%) with non-functional *FUT2*, and that about 25% of the Mexican population are lele (have a non-functional *FUT3*; these are largely Le a-b-). Using the phenotype screening approach, we will be able to estimate the rate and quickly find potentially unique non-functional alleles in the population. If no unique allele is found, the commonly used polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method will be used, targeting the common non-functional alleles that are well characterized in populations of European descent.

For mutations that are not easily detected by PCR-RFLP, the multiplex snap-shot genotyping method will be utilized. The advantage of this technique is that it allows detection of multiple mutations simultaneously. It would not be difficult to amplify the entire set of fucosyltransferase genes by this method, which may result in the discovery of inactivating mutations characteristic to the Mexican population which have not been previously reported in other populations. In our calicivirus studies, we also observed that in addition to Lewis and secretor genes, the ABO types also are involved in the calicivirus binding. Because there are a large number of non-functional alleles of the ABO gene families, it is impractical to characterize all of them. We will mainly focus on the ABO phenotyping; genotyping will be used only when unique individuals are found from phenotyping and NLV binding assays.

EPEC Project

The initial events in pathogenesis of multiple bacterial enteropathogens (*Shigella spp.*, enteropathogenic *E. coli* [EPEC], Shiga toxin-producing *E. coli* [STEC], *Yersinia spp.* and *Salmonella spp.*) involve sensing by the bacteria of intestinal epithelial cell milieu followed by upregulation of closely related secretory proteins (the type III secretory system) that transport virulence factors from the bacteria into the host cell. The injected virulence proteins then trigger a complex cascade of events that results in diarrhea. Each of these organisms produces an organelle that is critically important for initial bacteria-host contact and through which the virulence proteins are introduced into host cells. We hypothesize that these initial contact structures are major targets of the human milk protective machinery. The focus of this project will be to define the molecular basis and mechanism of action of human milk factors that protect infants by disrupting the function of these first contact organelles. We will utilize EPEC as a model for this group of pathogens.

A. Specific Aims

1. Determine the composition and biologic relevance of the human milk oligosaccharide(s) that prevent(s) initial EPEC contact with host cells.
2. Characterize the human erythrocyte receptor (histo-blood group antigen) for EPEC EspA and the relationship between erythrocyte phenotype and intestinal susceptibility to EPEC colonization and disease.
3. Characterize the mechanism of human lactoferrin protection against EPEC infection and disease.
4. Determine the relative contribution of human milk factors, including anti-EspA antibodies, lactoferrin, and oligosaccharides in protection from EPEC in tissue culture and animal models.
5. Determine the relative contribution of human milk factors, including anti-EspA antibodies, lactoferrin, and oligosaccharides in protection from EPEC in breastfed children.

B. Background and Significance

Epidemiology of EPEC. EPEC are among the most important pathogens infecting infants in the developing world. Infants who are not breastfed are at increased risk of death from EPEC.(1) EPEC cause both acute secretory diarrhea and persistent diarrhea in young children worldwide as documented in Bolivia (2) , India (3) , Brazil (4) , Argentina (5) , and the US. (6, 7) Each year EPEC are responsible for hundreds of thousands of deaths worldwide, mostly in infants and young children.(8)

EPEC classification/virulence genes/disease mechanism. EPEC originally were serogroup-defined E. coli associated with infantile diarrhea. As mechanisms of pathogenesis have been discovered, EPEC classification has come to be based on presence of specific genes. The strains currently classified as typical EPEC usually show the localized adherence (LA) pattern in tissue culture.(4) The LA phenotype is clearly related to human diarrheal disease although diffusely adhering strains also possess virulence genes.(9) Although less clearly related to diarrheal disease the atypical non-LA EPEC can, like the LA strains, cause accumulation of actin and induce the characteristic attaching effacing lesion.(10)

EPEC colonize the intestine in a distinctive way. They adhere to enterocytes causing distortion of the microvillus membrane with formation of a pedestal to which the bacteria are tightly adherent (figure 1). On transmission electron micrographs these changes are referred to as the "attaching effacing" lesion. Actin, α-actinin, myosin light chain, talin, and ezrin accumulation is associated with EPEC-induced morphologic alterations.(11, 12)

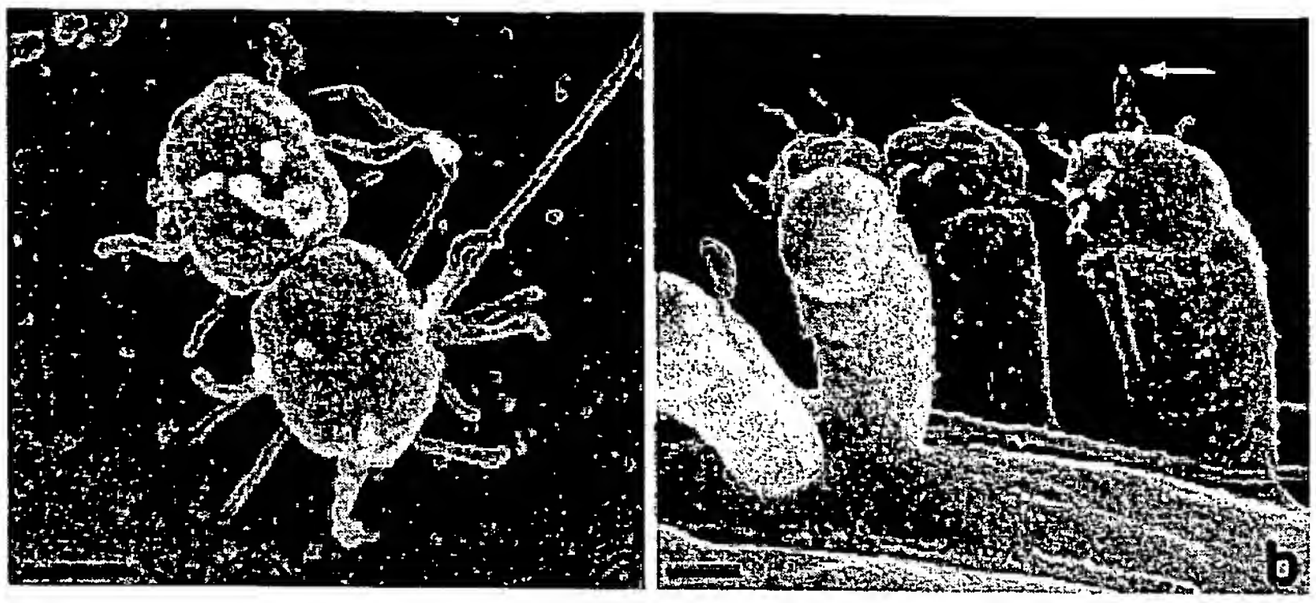


Figure 1: *Classic lesions of EPEC seen on scanning electron microscopy. The photo (a, left) shows EspA filaments on the surface of EPEC attached to HEL cells. The photo (b, right) shows EspA filaments on the surface of bacteria at the ends of pedestals.(13)*

The needle complex attachment organelle of EPEC. EPEC, like *Shigella, Salmonella, Citrobacter rodentium,* and *Yersinia,* make a needle complex that serves as a conduit for direct delivery of membrane lytic and intracellular effector molecules into mammalian cells. EPEC virulence proteins(14) are encoded in the chromosomal locus of enterocyte effacement (LEE).(15, 16) EscF, the EPEC needle, has homology to the needle in *Shigella* (MxiH), *Salmonella* (PrgI) and *Yersinia* (YscF). (17)

Multimers of secreted protein A (EspA) attach to EscF forming a tube-like structure extending from the end of the needle (seen in both photos of Figure 1). (13, 18, 19) The most important proteins transported via this tube are EspB, EspD, and the translocated intimin receptor "Tir". EspB is translocated into the cytosol and plasma membrane of host cells (13) where it forms pores on the host cell surface. EspD is a protein upregulator of secretion that is integrated into host cytoplasmic membranes where it serves as a permease. (20, 21) After EspB and EspD make a pore in the host cell, additional proteins are injected into the eukaryotic cell's cytosol through this opening.(22) Tir is injected into host cells and then integrated into the cytoplasmic membrane, (23-26) where it serves as a receptor for intimin, a bacterial outer membrane protein. Intimin is encoded by *eae;* it binds to Tir and thereby triggers intracellular signal transduction leading to cytoskeletal rearrangement including actin polymerization, effacement of microvilli and formation of actin rich pedestals. (25) Figure 2 shows a schematic representation of the series of events.

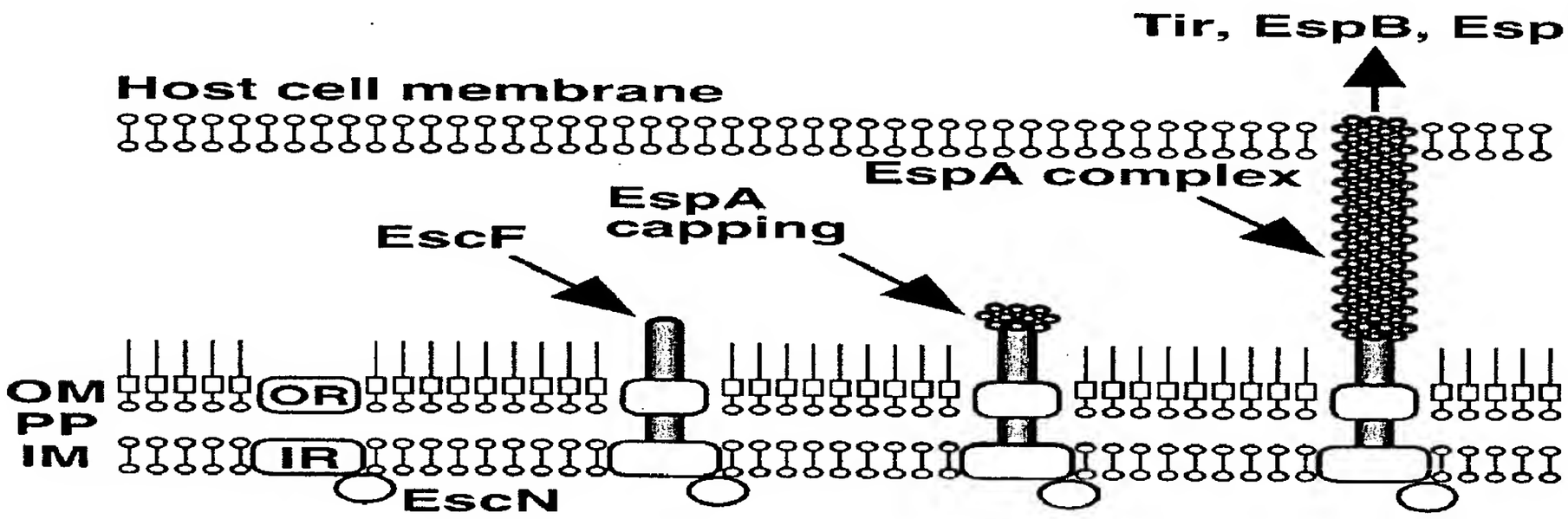


Figure 2*: Model of EPEC needle complex/EspA organelle interaction with mammalian host cells. Structures shown include the needle (EscF), the multimeric tube (EspA), the pump (EscN), the major secreted proteins (EspB, EspD, Tir), the outer membrane (OM), periplasmic space (PP), and inner membrane (IM) of the bacteria.[18]*

For most of the virulence proteins there are significant differences between the related proteins made by different organisms in this class of pathogens. For example, there are multiple variants of intimin (α1, α2, β1, β2, γ1, γ2, ε, and η). (10, 27-30) The large number of intimin types and the failure of antibodies to cross protect in animal models against different intimin types (31) makes anti-intimin unlikely to be the major mechanism of human milk protection. There are likewise significant variations in Tir, EspB, and BFP (bundle forming pilus). (30, 32) There are 3 variants each for EspA, EspB and Tir. There are eight variants (α1–3 and β1–5) for BFP. (30, 32) The lack of homology between Tir variants and the fact that Tir is secreted into host cells rather than exposed on the surface of the bacteria makes it unlikely that human milk will contain protective anti-Tir antibodies.

Unlike other virulence antigens, EspA is highly conserved. (32) EspA is surface expressed and multimeric. It has a critical role in initiating infection. Milk factors that disrupt its function might be particularly effective in blocking the cascade of events that result in infection. We hypothesize that the needle complex-EspA organelle is the target for protective antibodies, oligosaccharides, and lactoferrin.

Secretory immunity to virulence antigens of EPEC. EPEC infections are particularly common in children who have been weaned.(33, 34) Studies in Brazil and Egypt have shown that breastfeeding provides factors including sIgA (35) that protect infants from EPEC. (36, 37) Pooled colostrum has been shown to contain sIgA against EPEC virulence proteins. Milk protects against attachment by classic locally adherent EPEC. (38) In addition, chronic diarrhea related to EPEC infection responds to treatment with human colostrum. (39) Human colostrum from Brazilian women contains antibodies that block localized adherence of EPEC O111:H- to HeLa cells;(40) colostrum has been shown by Western blot to contain antibodies to a 94kDa outer membrane protein that may be intimin.(41-43) Evaluation of colostrum samples of four Brazilian women has shown(28) the presence of low titers of sIgA to intimin-□ and intimin-□□ there is variability in what portion of the C terminal 280 amino acid residues recognized. Using crude bacterial extracts, pools of Brazilian colostrum were shown to contain antibodies to all of the EPEC virulence antigens.(44) Anti-Tir antibodies have been found both in colostrum and serum of Brazilian children. Although anti-Tir antibodies are common in milk and serum, they do not appear to provide protective immunity to EPEC.(45) Colostrum from 21 Mexican women recognized EspA (76%), intimin (72%), BFP (52%), and EspB (57%).(46) Although it is clear that milk contains antibodies to multiple EPEC antigens, only anti-EspA occurs frequently enough to be a plausible candidate to explain protective immunity.

EPEC receptors and milk glycoconjugates. A number of surface antigens have been reported to play a role in EPEC pathogenesis. The potential host cell receptors for EPEC include β1-integrins(47) , N-acetylgalactosamine(48) , fucosylated oligosaccharides (38) , GM3 gangliosides(49) , the GalNAc β(1->4)Gal portion of asiaslo-GM1 and asialo-GM2(50) , N-acetyllactosamine sequences on glycoproteins(51, 52) , Lewis^{X}(52) , mucins(53) , and phosphatidylethanolamine(54, 55) In the rabbit model of EPEC infection, mucin inhibits binding(56) . However, none of the studies describing these potential receptors have been done using conditions that isolated the initial attachment step mediated by the needle complex associated EspA organelle. Some of these previous reports have used solid phase assays that focused only on intimin (47) or bundlin (54, 55) Others have used conditions for bacterial growth in which the initial attachment machinery is not expressed, (48, 53, 55, 57) or used cells in tissue culture (38, 48-53, 58) as the target so that the initial attachment was not specifically studied. Thus, the receptor for initial cell contact remains undefined. Since most of the putative receptors described above are carbohydrates, we hypothesize that milk oligosaccharides mimic epithelial cell glycoconjugate receptors that are involved in the initial attachment step, and that such oligosaccharides are potent inhibitors of EPEC colonization.

Effect of lactoferrin on bacterial enteropathogens. Lactoferrin, a major milk protein that has been postulated to contribute to protection from enteropathogens, acts on the bacterial cell surface to damage the outer membrane.(59, 60) Lactoferrin binds to the phosphate groups of lipid A. (61) The cationic portion of lactoferrin may disrupt interactions of lipopolysaccharide-associated cell surface proteins. There are two binding sites for lipopolysaccharide on lactoferrin: a high affinity site in the N terminal domain and a low affinity site in the C terminal domain.(62) Bactericidal activity of a pepsin cleaved peptide has been defined

as a segment near the N terminus consisting of a loop of 18 amino acids formed by disulfide bonds between residues 20 and 37.(63) Synthetic peptides corresponding to this cationic loop are rapidly bactericidal.(64, 65) Little is known about the effect of lactoferrin on EPEC. Lactoferrin binds to EPEC (66) and inhibits EPEC adherence to HeLa cells.(67)

C. Progress Report

Our previous publications include data supporting the role of human milk factors in protection from bacterial intestinal infections.(68-80) We have published data relevant to the immunology,(71, 72, 74, 81) pathophysiology,(82-96) genetic predisposition,(97) clinical manifestations,(98-100) diagnosis,(101-103) epidemiology,(104) etiology,(105-109) , therapy,(110, 111) and glycolipid metabolism(78, 97) of illness due to organisms that express the needle complex/type III secretory system. This subproject has previously focused on *Shigella* but because there have consistently been too few cases to answer some of the crucial questions, we have shifted to EPEC, a more common pathogen that shares important mechanisms with *Shigella* and for which critical issues related to humans can be addressed. New data relevant to the hypotheses described above and anticipated studies to be conducted during the renewal are summarized below:

Data relevant to Specific Aim 1: Determine the composition and biologic relevance of the human milk oligosaccharide that prevents EPEC initial contact with host cells. We have found that a preparation of total human milk oligosaccharides [TOS] was able to totally block contact hemolysis caused by EPEC O127:H6 E2348/69. The contact hemolysis assay isolates the first steps in pathogenesis (construction of the needle complex/multimeric EspA tube and presentation of EspB/EspD). Proteins acting beyond initial contact (Tir, EspF, EspG, intimin) do not play a role in this assay system.

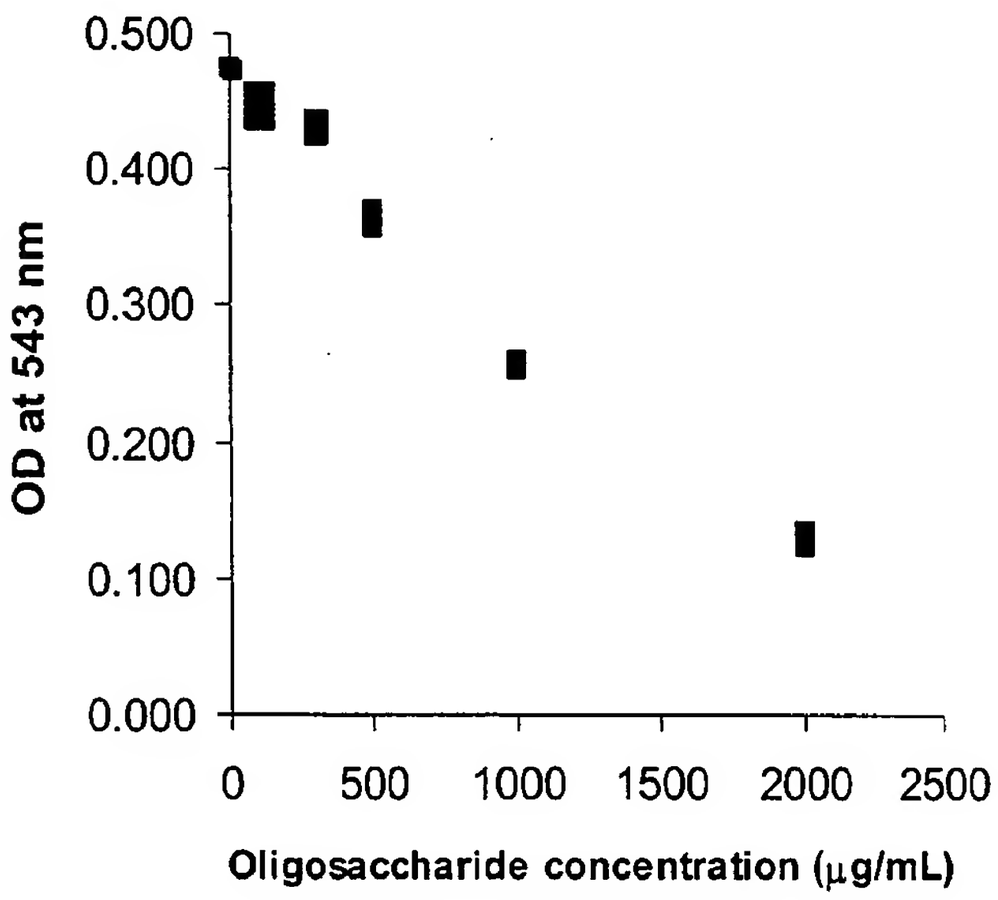


Figure 3: *Oligosaccharide inhibition of EPEC contact hemolysis. Human milk oligosaccharides block contact hemolysis in a dose dependent manner. OD_{543} of the supernatant for released hemoglobin is measured after a 6hr incubation of erythrocytes with the bacteria grown to log phase in DMEM-HEPES (n=5 experiments). Osmolarity of these sugar solutions is too low to account for protection from hemolysis.*

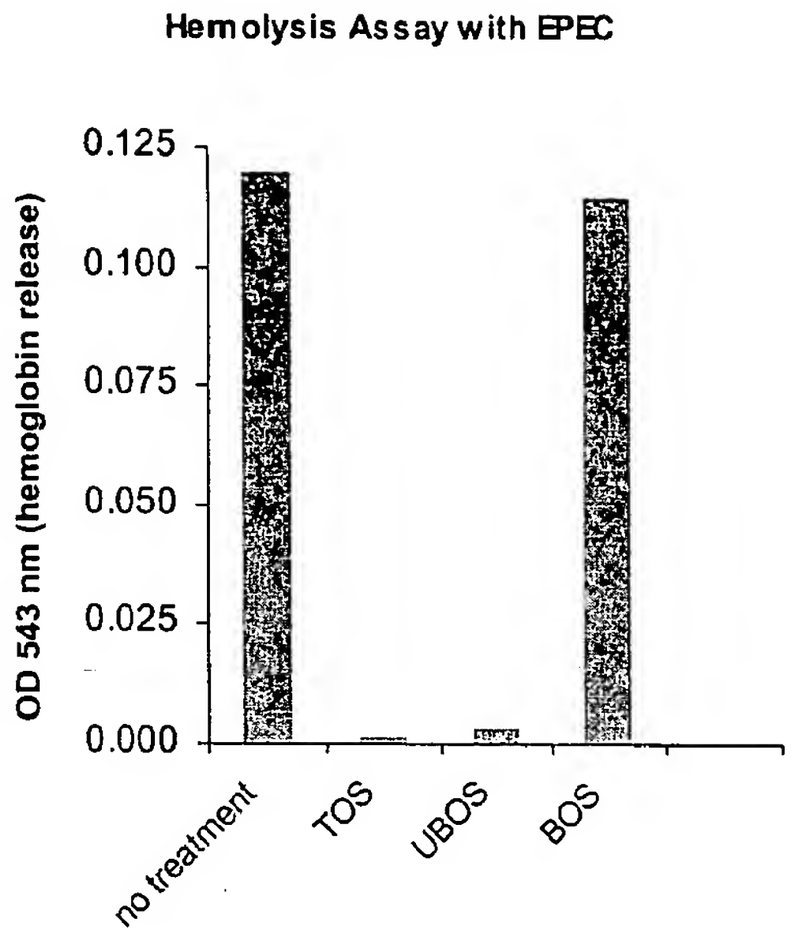


Figure 4: *Inhibition of EPEC contact hem-olysis by partially purified oligosaccharides. The column far left shows hemolysis induced by EPEC in the absence of milk oligosaccharides. The total oligosac-charide fraction [TOS] totally blocked contact hemolysis. The fraction of oligosaccharides that bound to a Ulex europaeus column [BOS] did not block hemolysis while the fraction that passed through that column without binding [UBOS] contained the anti-hemolytic factor*

The significance of these studies is that they demonstrate that there is an oligosaccharide(s) in milk that can prevent the initial interaction of EPEC with host cells.

Data relevant to Specific Aim 2: Characterize the human erythrocyte receptor for EPEC EspA and the relationship between erythrocyte phenotype and susceptibility to EPEC colonization and disease. Healthy blood donors have been studied repeatedly over a four-month period by the contact hemolysis assays. Unexpectedly we found that there was a good correlation between erythrocyte donor and susceptibility to hemolysis induced by E2348/69 ($r = 0.9109$, $n=5$, $p < 0.02$). The significance of these preliminary data is that they suggest that expression of the erythrocyte receptor recognized on the EspA organelle varies among individuals. Since erythrocyte carbohydrate receptors are often mimicked on the gut, such variability in erythrocyte receptor expression may well relate to variability in susceptibility to EPEC. Pigs differ in their expression of small intestinal receptors for *E. coli* K88 fimbrial antigenic variants.(113) Whether humans vary in a similar fashion in their expression of EPEC intestinal receptors is not yet known. Data presented elsewhere in this grant support this concept for other pathogens.

Data relevant to Specific Aim 3: Characterize the mechanism of human lactoferrin protection against EPEC infection and disease. We have been investigating the role of lactoferrin in impairing virulence of enteropathogens using a *S. flexneri* model. Rabbits were infected with *S. flexneri* with or without lactoferrin in their intestinal tracts. Gross evidence of inflammatory changes developed significantly more often in rabbits infected without lactoferrin (22/33 [67%]) than in those infected with lactoferrin exposure (4/35 (11%)) ($p < 0.001$). The degree of microscopic inflammation interpreted by a blinded observer was significantly less in the infected animals treated with lactoferrin than in those not given lactoferrin (inflammation score 2.1 ± 0.3 versus 3.8 ± 0.3 (mean ± SEM), $p < 0.001$).

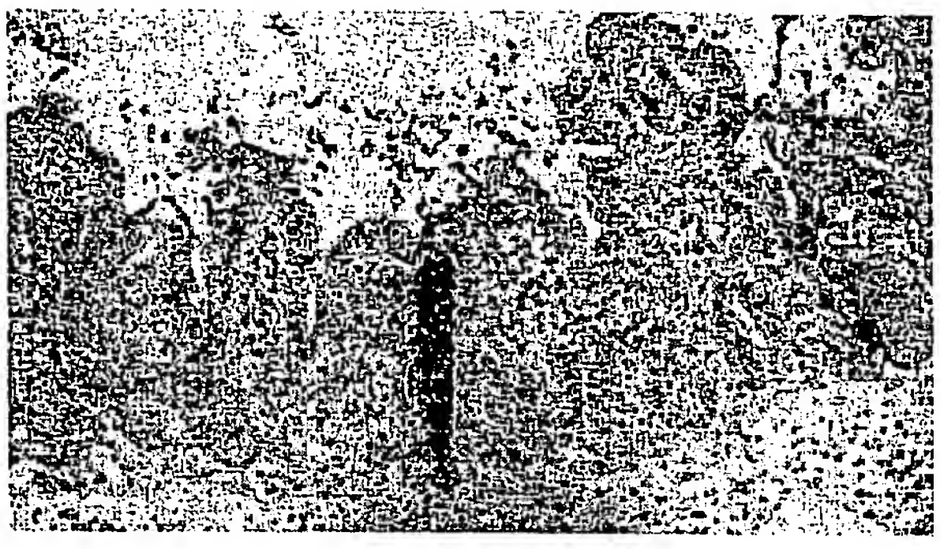


Figure 5: *Rabbit intestine in a control animal infected with S. flexneri but not given lactoferrin. Note severe mucosal hemorrhage, submucosal edema, and villous blunting and atrophy.*

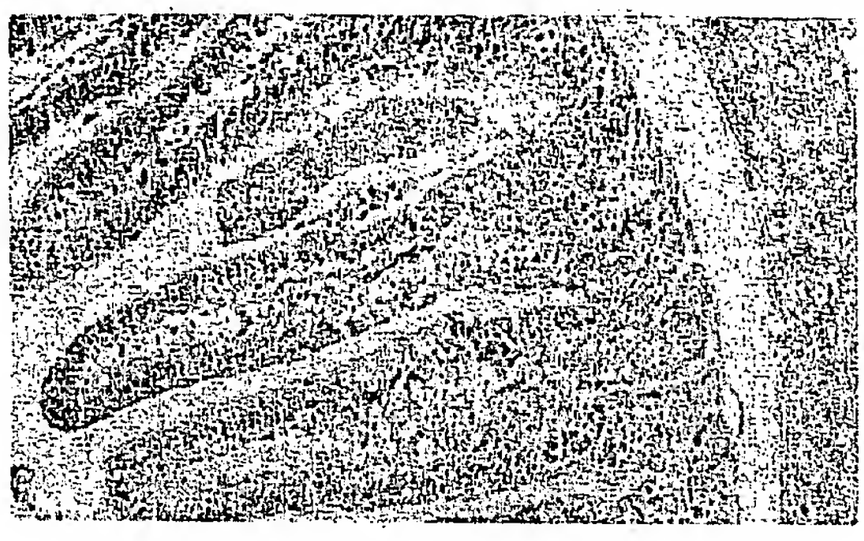


Figure 6: *Rabbit infected with S. flexneri and treated with lactoferrin. Note absence of inflammatory changes.*

These observations could reflect lactoferrin-mediated bacteriostasis, impaired virulence, impaired attachment. To investigate the possibility that *S. flexneri* might be inhibited in its growth by lactoferrin, we evaluated growth curves with lactoferrin present in a range of concentrations.

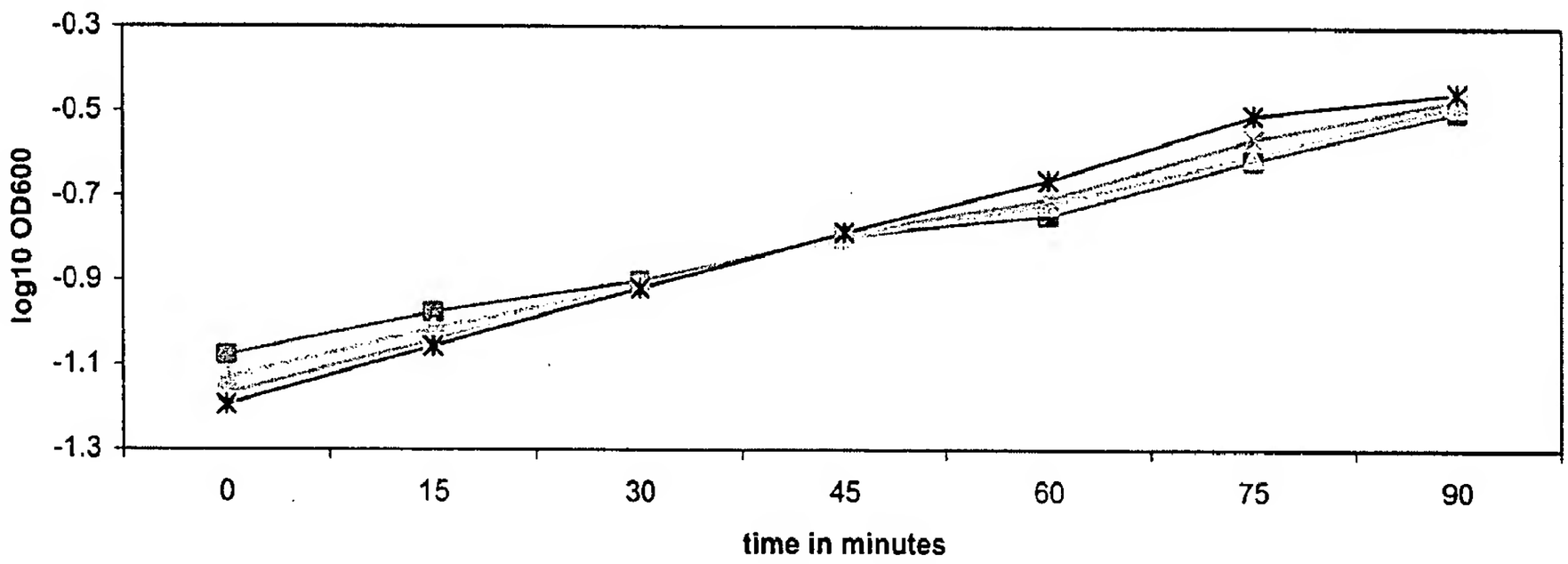


Figure 7: *Growth curve of S. flexneri in the presence of various concentrations of lactoferrin. The figure shows log phase growth (as indicated by change in OD600) of S. flexneri M90T in Luria Bertani broth containing various concentrations of lactoferrin (0.125mM ▪, 0.062mM ◦, 0.012mM ×, and 0mM ×). Lactoferrin was not cidal or static when adequate iron was made available for bacterial growth. There was no difference in bacterial growth rate related to presence or absence of lactoferrin.*

Lactoferrin pretreatment did not block attachment of *Shigella flexneri* to cells in multiple model systems: sheep erythrocytes, tissue culture (HeLa cells), intact gut (mouse intestine), or isolated intestinal cells (rabbit) (data not shown). We therefore investigated the possibility that lactoferrin impaired bacterial virulence. For these studies we used an in vitro invasion model system. In a HeLa invasion model, lactoferrin impaired the ability of the organism to invade as shown in figure 8 (below).

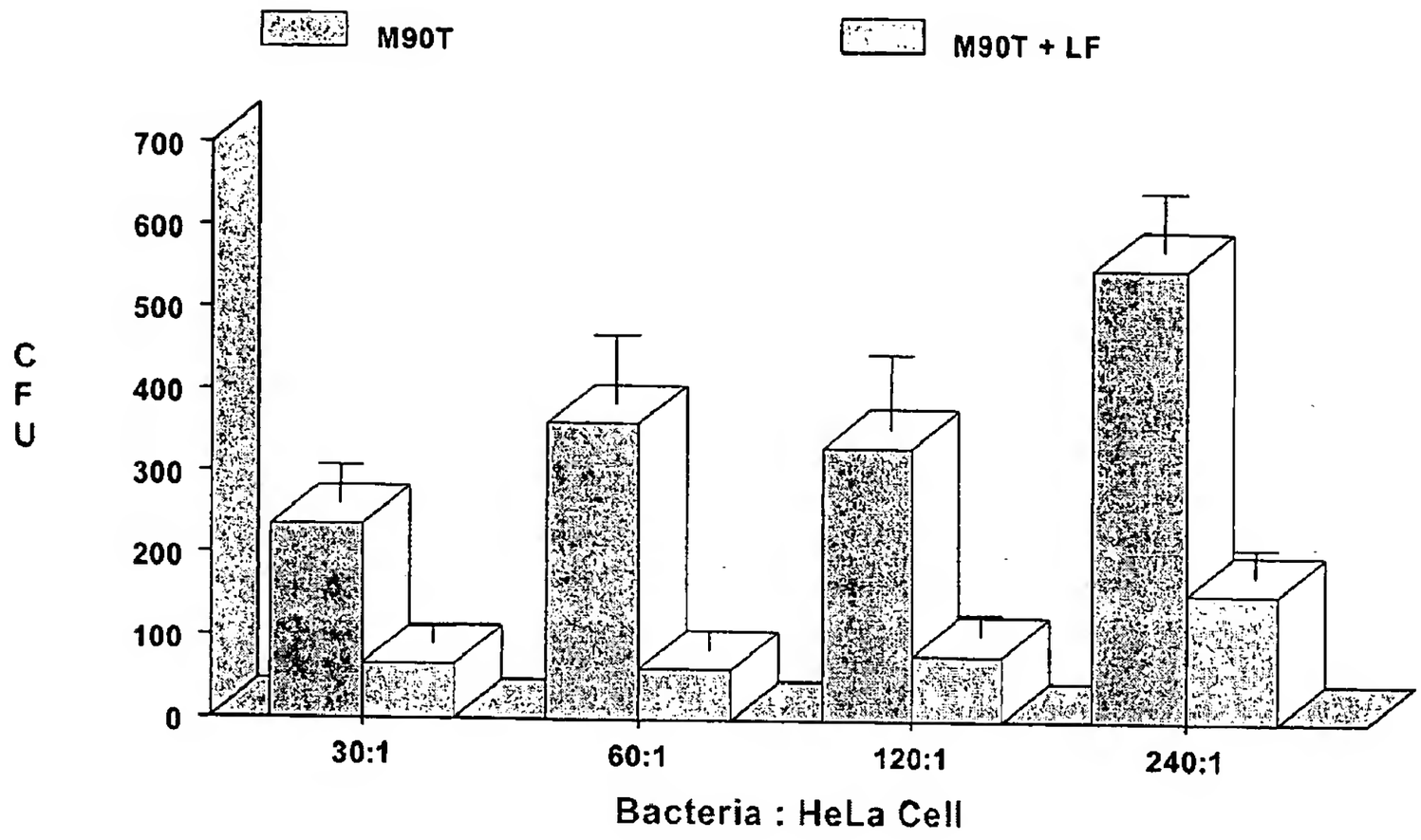


***Figure 8**: HeLa cell invasion by Shigella flexneri serotype 5 strain M90T. The effect of lactoferrin pre-incubation is shown at various bacteria to target cell ratios. Data represent means ± SEM for six to ten experiments at each ratio. Dark bars (orange) represent mean colony forming units (CFU) in the absence of lactoferrin while lighter bars (green) show CFU after the bacteria have been exposed to lactoferrin. The difference in CFU at each bacteria to target ratio was significant (p <0.03-<0.001). These findings suggest disruption of the invasion plasmid antigen mediated uptake of bacteria.*

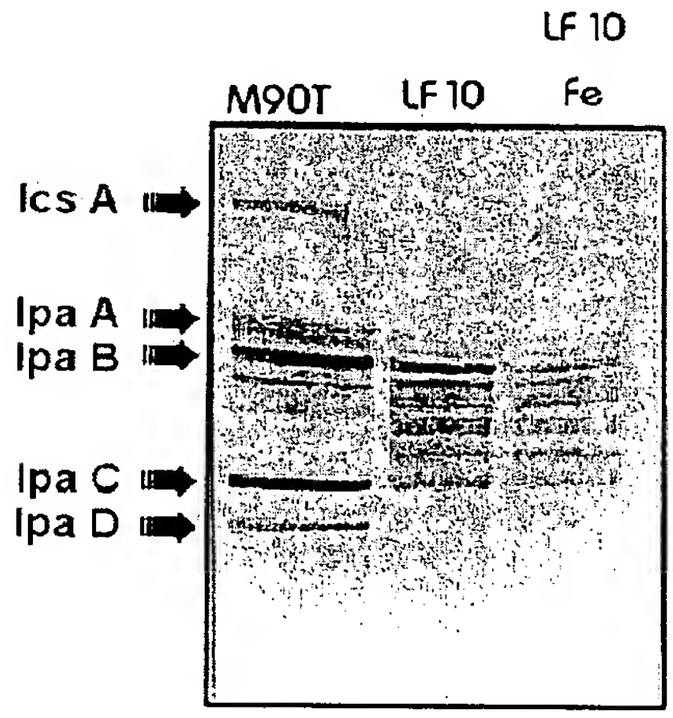


***Figure 9**: Immunoblot of proteins released from S. flexneri by lactoferrin treatment. This immunoblot (left) shows that a convalescent serum reacts with IcsA, IpaA, the two isoforms of IpaB, IpaC, and IpaD in an invasion plasmid antigen preparation not treated with lactoferrin (left lane "M90T"). Treatment of M90T for 1hr with lactoferrin (10mg/mL [0.125mM]) causes degradation and release into the supernatant of IpaB (middle lane) while ferric iron saturation ($FeCl_3$ 2:1 molar ratio) of lactoferrin does not change the lactoferrin-induced release and breakdown of IpaB (LF10/Fe, right lane).*

This release is lactoferrin concentration dependent as shown in the following immunoblot.

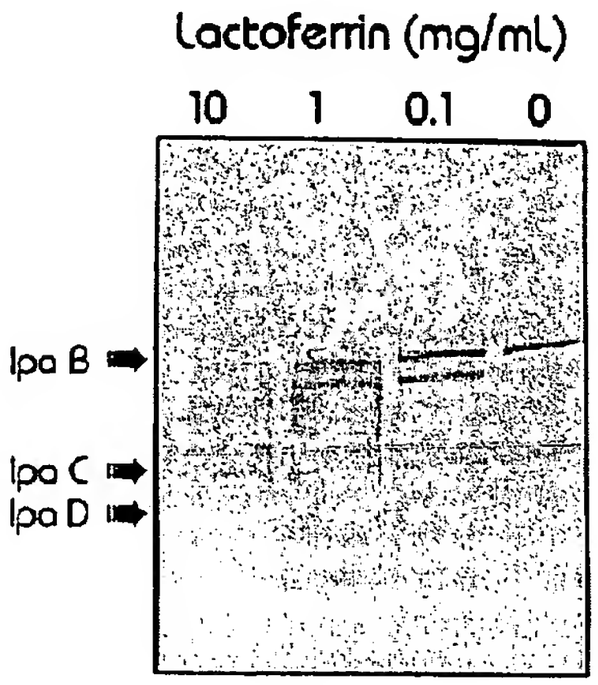


***Figure 10**: Concentration dependent release and degradation of S. flexneri virulence antigens. The immunoblot (left) shows the release and breakdown of IpaB from S. flexneri incubated for one hour at 37C in buffer alone (right lane) or increasing concentrations of recombinant human lactoferrin 0.1 to 10mg/mL [0.00125 to 0.125mM]. The location that IpaD would have been seen if it were released is also shown. The major protein released and degraded was IpaB; faint bands suggest that some IpaC or IpaD was also released and degraded. Immunoblots using monoclonal antibodies to IpaB, IpaC, and IpaD confirmed degradation of IpaB and IpaC only. (not shown)*

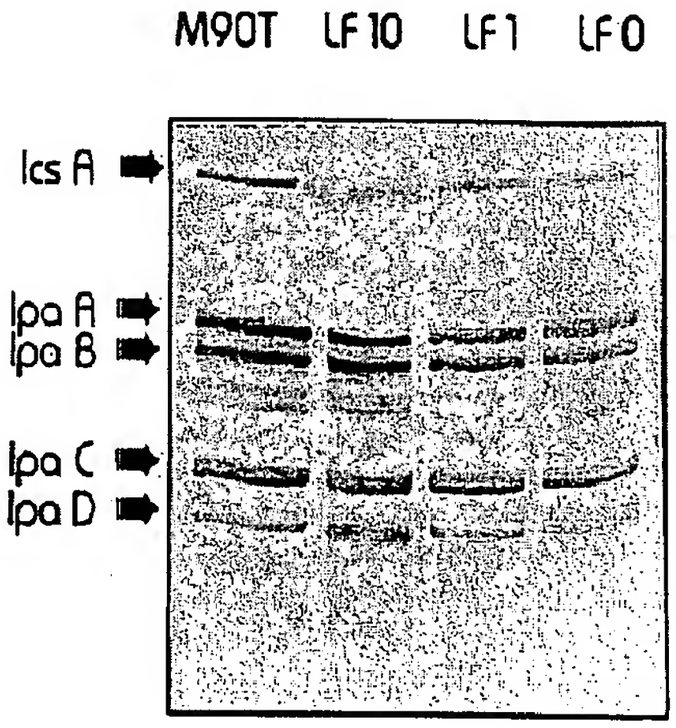


***Figure 11**: Lactoferrin does not degrade the virulence antigens after they have secreted by S. flexneri . A cell-free invasion plasmid antigen preparation was treated for one hour at 37C with lactoferrin (0.125mM). The left lane of Figure 11 shows the cell free invasion plasmid antigen extract without lactoferrin pre-incubation. The three lanes to the right show this preparation incubated with 0-10mg/mL of lactoferrin. Lactoferrin acts at the bacterial cell surface rather than on the invasion plasmid antigens after they have been secreted.*

Thus, the mechanism by which lactoferrin blocks invasion is disruption of the bacterial cell surface of invasion antigens secreted via the *Shigella* needle complex. There is loss and degradation of two major virulence proteins, invasion plasmid antigen B [IpaB] and to a much lesser extent, invasion plasmid antigen C, [IpaC]. Other virulence proteins (IcsA, IpaA, IpaD) were not affected. Of the five major virulence antigens only IpaB and IpaC are tightly associated with the needle complex.(114) PSI-BLAST searches show that there is weak homology between the *Shigella* needle protein (MxiH) and lactoferrin-binding protein A (AF049349) and lactoferrin binding protein B of *N. meningitides* (AF031432). To further test the hypothesis that the needle complex might be a target for lactoferrin, we have begun investigations into the effect of lactoferrin on a needle complex dependent activity (contact hemolysis) of EPEC.

Lactoferrin blocks the initial attachment step of EPEC

We found (Figure 12) that human recombinant lactoferrin blocks contact hemolysis caused by EPEC E2348/69 in a dose dependent fashion. Blockade of contact hemolysis implies that for EPEC as for *Shigella*, lactoferrin acts at the initial step of needle complex dependent host cell engagement.

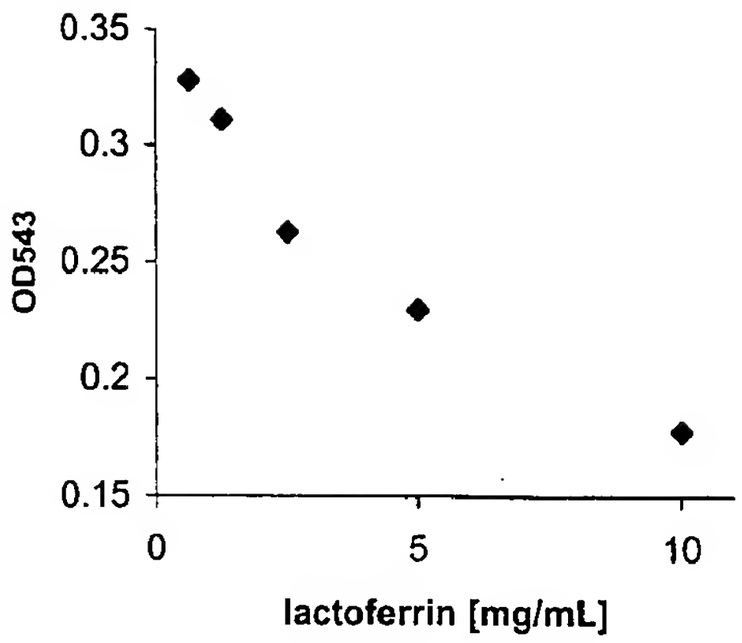


***Figure 12**: Lactoferrin decreases contact hemolysis caused by EPEC O127:H6 strain E2348/69. With increasing concentrations of lactoferrin the ability of EPEC E2348/69 to lyse erythrocytes (as indicated by OD543) is blocked. Hemolysis in this assay was determined at the end of a six hour incubation of the human erythrocytes with the bacteria (n=3 experiments)*

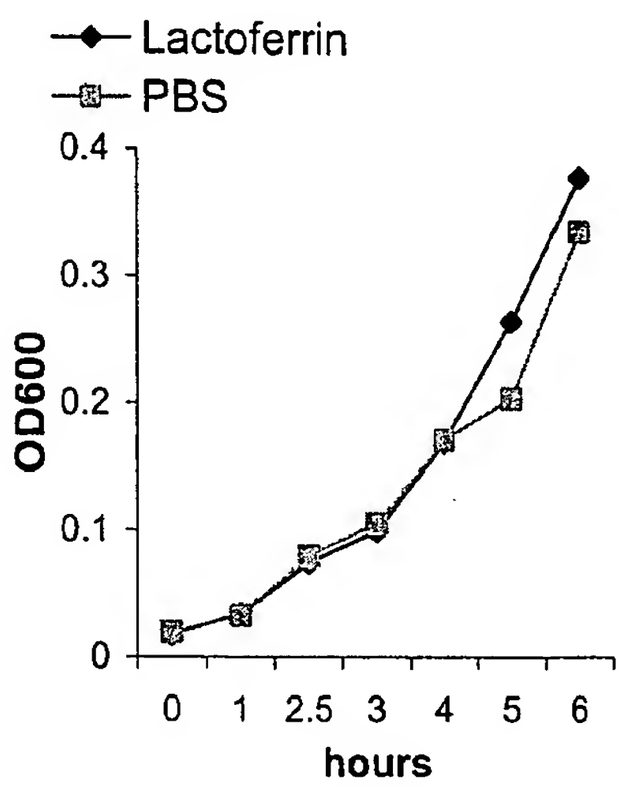


***Figure 13**: Effect of lactoferrin on growth of EPEC O127:H6 strain E2348/69. Lactoferrin did not impair growth (indicated by change in OD600) as shown (above) when the organisms are grown in rich media (DMEM HEPES). Lactoferrin in PBS or PBS was added to the culture media prior to inoculation with bacteria.*

The initial step in cell contact involves EPEC making a needle complex with EspA multimers forming filaments at the end of the needle (EscF) and subsequent injection of EspB and EspD into host cells to lyse cell membrane (as shown in Figure 2). We therefore have investigated the effect of lactoferrin treatment on the secretion of the virulence proteins using EIA (monoclonal antibody based for EspB and EspD and affinity purified antibody based for EspA). Bacteria were grown with lactoferrin in the absence of mammalian target cells but presence of media conditions (DMEM/HEPES) known to stimulate secretion of the needle complex/EspA organelle.

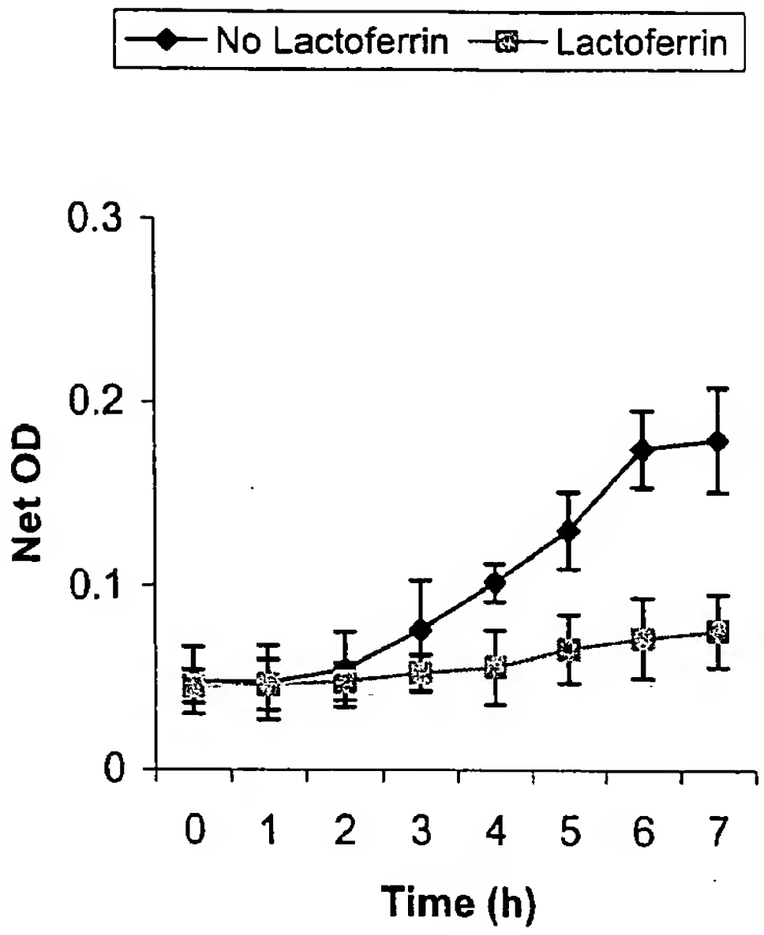


***Figure 14**: Effect of lactoferrin on EspA secretion in the absence of mammalian target cells. The secretion of EspA by EPEC E2348/69 is blocked by lactoferrin (0.125mM) in the growth medium (right). Data represent mean ±sem for three separate experiments. An immunoblot (not shown) confirmed the EIA findings. Since a BLAST search shows that EspA has homology to lactoferrin binding protein of Neisseria meningitidis [AF123380], the mechanism of this effect could be interference in formation of the EspA multimer by lactoferrin binding monomers and blocking assembly of the organelle.*

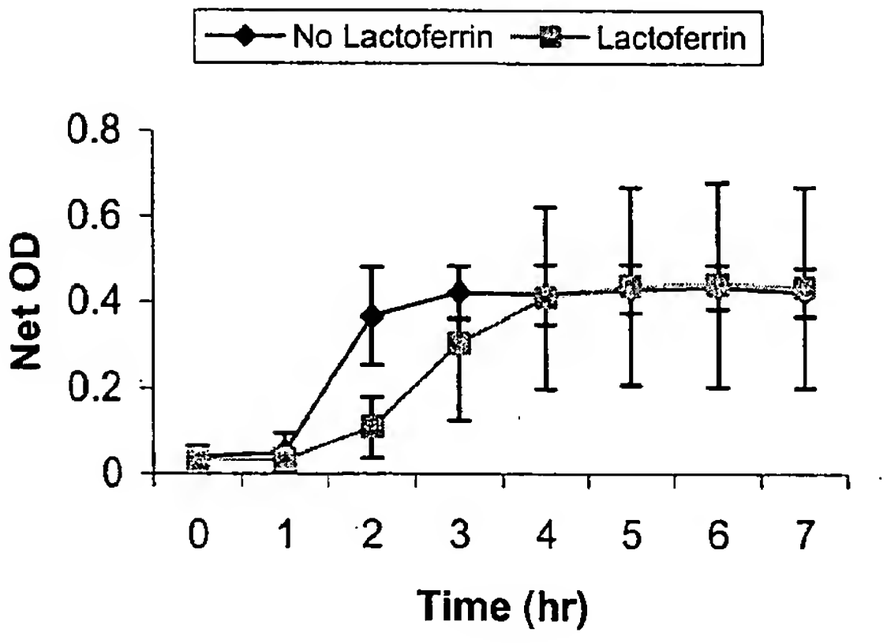


***Figure 15**: Effect of lactoferrin on EspD secretion by EPEC E2348/69 in the absence of mammalian target cells.*

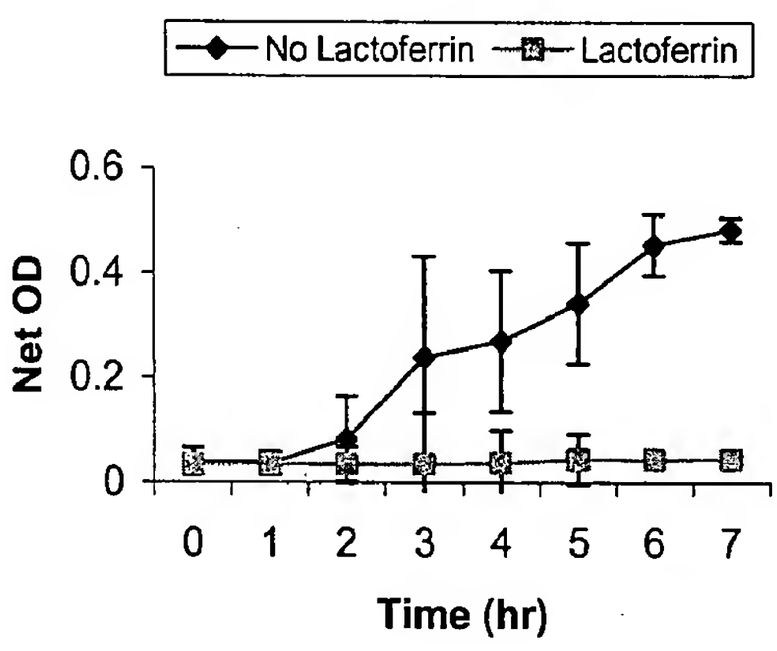


***Figure 16**: Effect of lactoferrin on EspB secretion by EPEC E2348/69 in the absence of target cells*

At present we are uncertain of the explanation for delayed secretion in the case of EspD (Figure 15, above left) and complete blockade in the case of EspB (Figure 16, above right). We suspect that EspD can be secreted by an EspA-independent mechanism while EspB secretion is totally dependent on formation of a functional needle complex/EspA filament organelle. The data above imply that lactoferrin may act at an early step in construction of the needle complex/EspA organelle. Thus it is not clear yet whether the effect of lactoferrin is on EspA or on a protein required earlier in the formation of the needle complex. It is also possible that lactoferrin blocks interaction of the needle complex with LPS. In summary, the significance of the above findings related to Shigella and EPEC is that they suggest a fundamental action of lactoferrin may be to disrupt the function/formation of the needle complex, an organelle whose role is central to the pathophysiology of EPEC, STEC, Shigella, Salmonella, and Yersinia. As such, they suggest a central role for lactoferrin.

Data relevant to Specific Aims 4 and 5: Determine the relative contribution of human milk factors, including antibodies, lactoferrin, and oligosaccharides in protection from EPEC in tissue culture, animal models and

breastfed children. EPEC infections are among the most common infections in the children of San Pedro Martir, after rotavirus, caliciviruses, and campylo-bacteriosis. By the end of the current study period [March 2003] we conservatively project that there will be over 30 breastfed children with symptomatic, non-coinfected EPEC infections available for study.

EIA for detection of EPEC specific sIgA. We have established assays for the relevant surface expressed antigens. In general, an OD $\geq$0.1 above background correlates with immunoblot detected antibodies. We have found that for many of the common EPEC LPS types sIgA is infrequently found in milk. Of the major virulence proteins only EspA is recognized by nearly all milk samples.

Table 1 (below) shows the frequency of detectable sIgA in milk of 73 different Mexican women at various points during lactation in a cross sectional study. $Int_{C280\gamma}$ is the extracellular domain of intimin gamma.

Table 1: Prevalence of milk sIgA against EPEC/STEC virulence proteins and LPS

N	EspA	EspB	$Int_{C280\gamma}$	O26 LPS	O127 LPS	O55 LPS	O111 LPS	O128 LPS
73	71 (97%)	32 (44%)	20 (28%)	58 (79%)	42 (58%)	35 (48%)	44 (60%)	7 (10%)

In serial samples, we found that anti-EspA persisted at easily detectable levels for more than one year of lactation. The titer of anti-EspA varies in individual milk samples from 1:20 to 1:1280. *Anti-EspA occurs frequently enough and persists long enough to be plausible as a dominant protective antibody.* We predict that the amount of antibody to EspA will relate directly to symptoms during EPEC infection of breastfed infants. Because of the high frequency of anti-EspA, we have developed an affinity column method for purification of antigen specific sIgA. Specific activity (anti-EspA/µg sIgA) increased 300-fold.

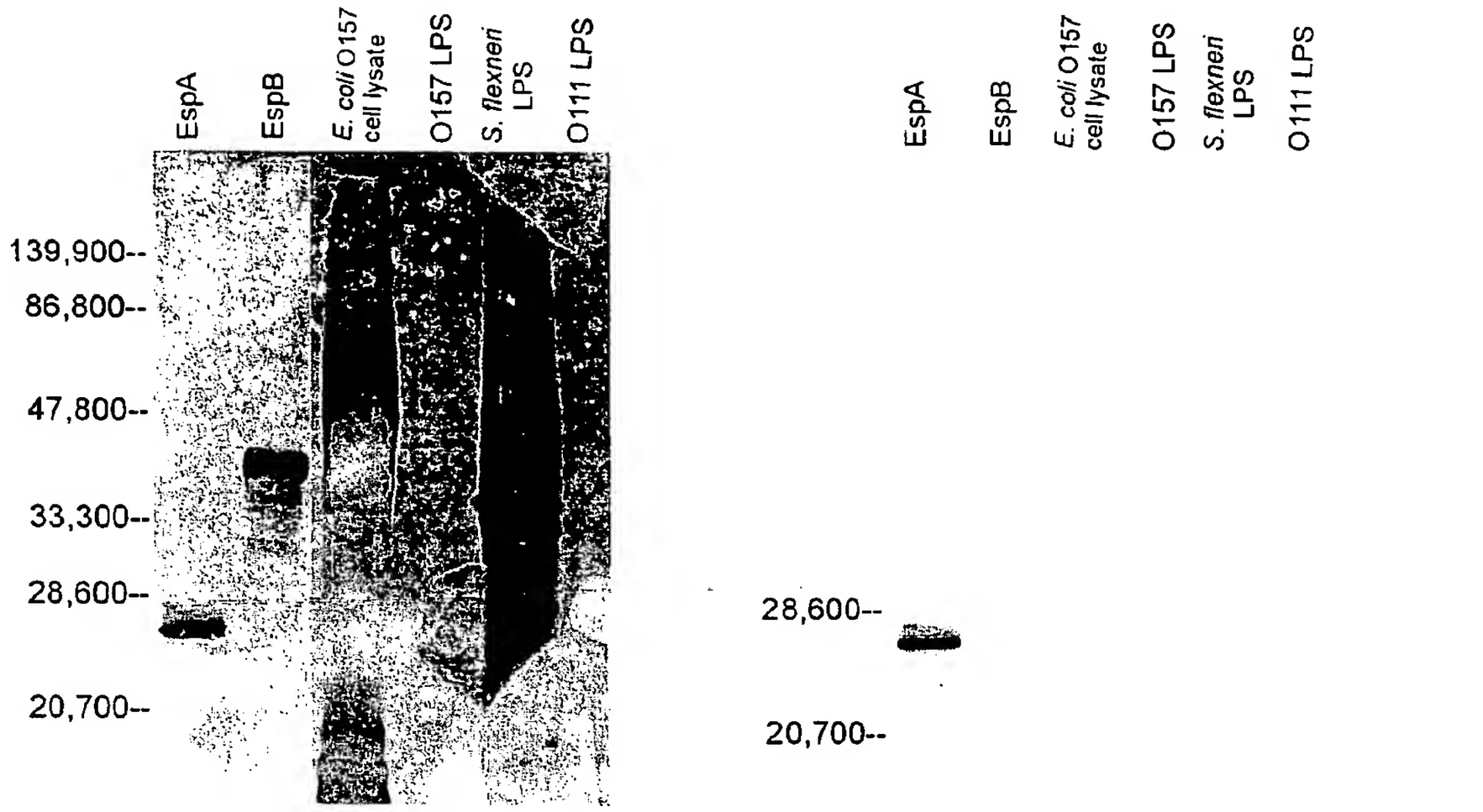
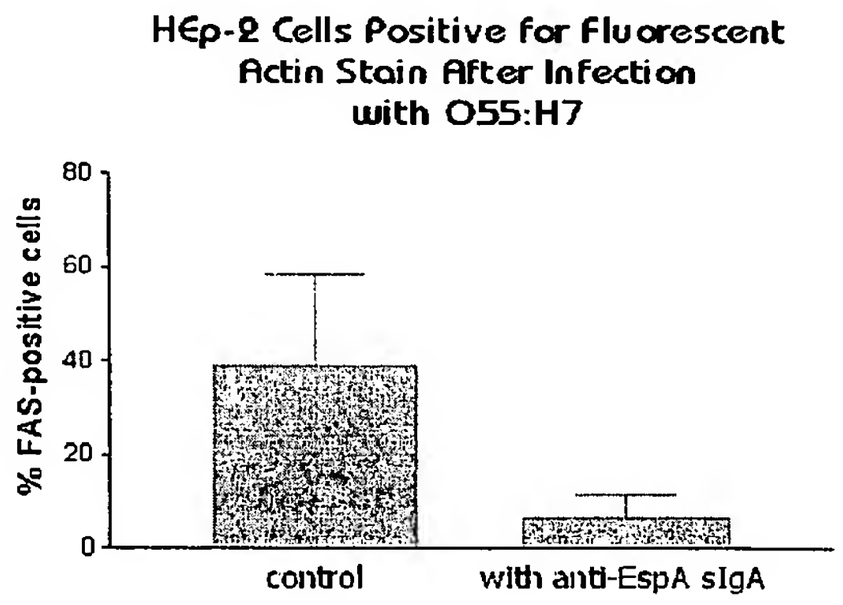


***Figure 17**: Western blot of pooled milk reacted with various bacterial antigens (left) and affinity purified EspA-specific sIgA reacted with the same antigens (right). The pooled human milk sIgA (above left) prior to affinity purification reacted with purified histidine tagged EspA, purified histidine tagged EspB, E. coli O157 whole cell lysate, O157 LPS, S. flexneri 5 LPS, and O111 LPS. EspA and EspB were not detected in the O157 whole cell lysate because the organisms were not grown in conditions (DMEM/HEPES) that stimulate expression of these proteins. [lane 4, left]. The affinity purified antibody reacted only with EspA (right, lane 1). EIA using the affinity purified anti-EspA sIgA confirmed that only EspA was recognized; there was no reaction with EspB, O157 LPS, or S. flexneri serotype 5 LPS. To determine whether this affinity purified antibody might be protective, we evaluated its ability to block EPEC triggering of actin polymerization in HEp2 cells.*

***Figure 18:** Fluorescent Actin Stain [FAS] assay in presence/absence of affinity purified anti-EspA. The purified anti-EspA sIgA was tested for ability to block adherence and polymerization of actin in a HEp2 fluorescent actin stain assay (right). EPEC O55:H7 strain O660/79, an EPEC wild type that does not contain bundle-forming pilus was tested in this assay. Preincubation with anti-EspA significantly reduced FAS+ cells (39±19 to 6±5% [counts by blinded observer], p<0.05).*



Similar results were found with STEC O157:H7 strain 86-24: FAS+ 34±7% in media alone vs 0.6±0.1% in presence of anti-EspA sIgA (p<0.02). In contrast to anti-EspA, affinity purified antibody to an irrelevant antigen (anti-LPS of *S. flexneri* serotype 5) did not block FAS induced by these bacteria. Affinity purified anti-EspA antibody did not cause a decrease in CFU. This mechanism by which affinity purified anti-EspA antibody blocked development of actin polymerization in Hep2 cells was next studied using the STEC strain as a model.

After contact with host cells, virulence proteins EspB and EspD are transported via EspA filaments to the mammalian cell surface where they form a pore. Actin polymerization occurs after Tir is injected through the hole made by EspB and EspD. Tir is inserted into the host cell membrane where it is engaged by the bacterial surface protein intimin; this interaction triggers actin polymerization.

We found that affinity purified anti-EspA sIgA decreased bacterial attachment to HEp2 cells by 85% ($p < 0.01$). However, this was not the only effect of this antibody. We also evaluated the secretory function of the needle complex/EspA organelle. We measured release into medium of virulence proteins normally delivered through the EspA multimeric organelle.

The following figures show secretion of these proteins in the presence of media conditions that stimulate EspB/D secretion (DMEM/HEPES) but in the absence of HEp2 cells. Anti-EspA blunts secretion of the pore forming proteins EspB and EspD. Figure 19 shows mean±sem at various time points for EspB and EspD secretion from six experiments using an EspA secreting strain (STEC O157:H7 strain 86-24); p ranges from <0.01 to <0.05 for the various time points for both EspB and EspD.

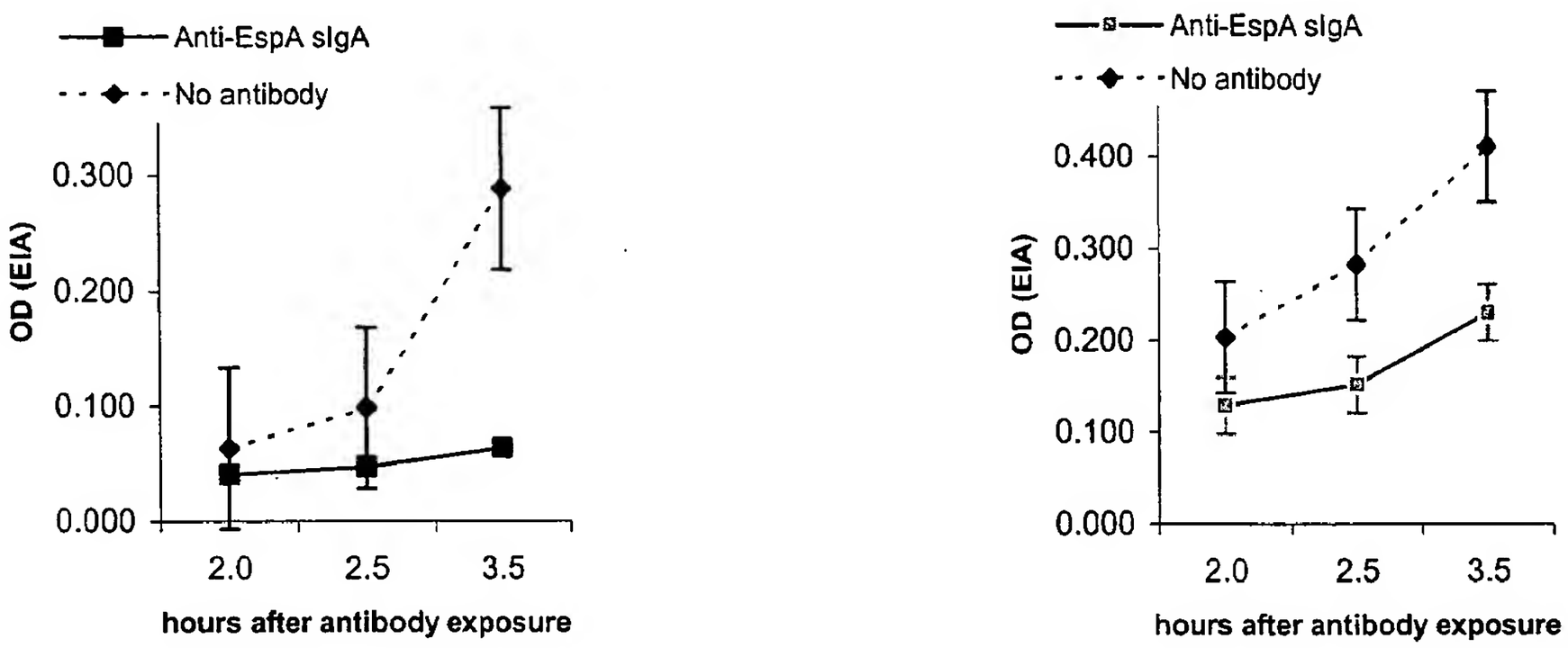


***Figure 19**: Effect of affinity purified anti-EspA sIgA on secretion of EspB (left) and EspD (right) in the absence of mammalian target cells. These finding suggest a major role for antibody to EspA in blocking the initial steps critical to development of the attaching effacing lesion of both EPEC and STEC.*

In summary, our preliminary data suggest that multiple milk protective factors (oligosaccharides, lactoferrin, sIgA) share as their common target the mechanism responsible for first contact between the bacteria and host cells. The following aims are intended to further elucidate the role and mechanism of each of these factors.

D. Research Design and Methods

RESEARCH DESIGN

Specific Aim 1: Determine the composition and biologic relevance of the human milk oligosaccharide that prevents initial EPEC contact with host cells.

Our preliminary data show that human milk oligosaccharides block the initial step in bacteria-host interaction as indicated by blockade of contact hemolysis induced by EPEC O127:H6 strain E2348/69. We will determine whether this finding is due to a single oligosaccharide or a motif common to many oligosaccharides. The Glycobiology Core of Dr. Newburg will purify oligosaccharides for evaluation in our

laboratory. We will focus initially on E2348/69 and subsequently determine whether other EPEC are inhibited by the same oligosaccharide(s). We anticipate using the contact hemolysis and HEp2 FAS assays for these studies.

Anticipated results, data analysis, and alternate approaches: The oligosaccharide(s) we define may be an analog of one of the putative receptors already described. If so we will have defined initial attachment as the step in pathogenesis that is relevant to that receptor and the milk oligosaccharide. The purification of oligosaccharides and alternate approaches to isolation of sugars is as outlined by Dr. Newburg in the Glycobiology Core. This approach is likely to yield relevant oligosaccharide fractions for investigation given our previous experience with purifying such compounds. It is likely that the contact hemolysis data will yield clear results given our preliminary studies and given that it is the standard assay for study of the initial events in EPEC attachment. The hemolysis data will be analyzed by ANOVA. HEp-2 FAS studies will be done to confirm that blocking the attachment step blocks downstream events as indicated by actin polymerization; these assays will be read by a blinded observer and analyzed for percent FAS+ cells by ANOVA. It would not be surprising if there were several glycoconjugates that protect against different EPEC variants of EspA. If we are successful in rapidly defining the specific oligosaccharide(s), we will define the frequency and amounts present in human milk in different Mexican women over time.

Specific Aim 2: Characterize the human erythrocyte receptor (histo-blood group antigen) for EPEC EspA and the relationship between erythrocyte phenotype and susceptibility to EPEC colonization and disease.

We have found that there are striking differences among individual humans in the susceptibility of their erythrocytes to the initial step in EPEC attachment as indicated by contact hemolysis. We hypothesize that these erythrocyte receptor differences reflect qualitative or quantitative differences in gut receptors for EPEC and as such may relate to susceptibility to colonization and disease. We will evaluate this hypothesis by three approaches.

First, erythrocytes from Mexican infants who have or have not been ill with symptomatic EPEC infection will be compared for susceptibility to contact hemolysis. Infants from the prospective 1998-2003 cohort will be used for these studies. Contact hemolysis will be studied using EPEC E2348/69. The studies to determine individual variability in infant erythrocyte susceptibility will be done in collaboration with the Epidemiology Core.

Second, we will partially characterize the human erythrocyte receptor for EspAα. Since a milk oligosaccharide inhibits E2348/69 EspAα-mediated contact hemolysis, we will determine the ability of simple sugars (D-galactose, N-acetylglucosamine, N-acetylgalactosamine, L-fucose, D-mannose, D-glucose, lactose] to inhibit E2348/69 mediated contact hemolysis using erythrocytes that are highly susceptible. Subsequently, erythrocytes will be treated with various glycosidases to determine whether contact hemolysis can be blocked by removing specific sugars. Lectin or antibody-mediated hemagglutination will be used to monitor adequacy of glycosidase treatments. Antibody mediated agglutination using commercially available antisera to all of the common blood group antigens will be used to determine whether susceptibility of red cells relates to a known histo-blood group antigen. We predict that the E2348/69 receptor will be carbohydrate motif related to the carbohydrate(s) defined in Specific Aim 1.

Third, we will define the relationship between erythrocyte receptors and susceptibility in an animal model of EPEC infection (*Citrobacter rodentium* in mice). We will determine susceptibility to contact hemolysis in various inbred strains of mice. We anticipate studying A/J, BALB/c, DBA/2, SJL/J, FVB, CBA/J, C57BL/6, and C3H mice. These strains have been chosen because they are inbred (so the erythrocyte surface antigens should be constant) and because timed pregnant females are available commercially. The plan is to determine strain-related variability in *C. rodentium*–induced contact hemolysis and relate it to

susceptibility to colonic hyperplasia. We will partially characterize the erythrocyte receptors as described above for human erythrocytes.

Anticipated results, data analysis, and alternate approaches: The alternate approaches for addressing the hypothesis that erythrocyte receptors are related to gut receptors for EPEC include studies of infected children, phenotypic erythrocyte characterization and a mouse model. We anticipate that with these multiple approaches, it is likely that the hypothesis will be adequately tested.

The individual variability in susceptibility of infants to contact hemolysis induced by EspAα will be analyzed by Student t test (comparing ill vs. well EPEC culture positive breastfed infants). Since more than one receptor may be relevant to EPEC initial contact and colonization, we will characterize the EspA subtypes of the infants isolates. Those infected with EspAα producers will be analyzed separately from those infected with EspAβ producers. It is anticipated that if more than one receptor is relevant to initial contact, those infected with an EspAα producer will have E2348/69 induced hemolysis that relates well to symptom status, while those infected with an EspAβ producing strain will not. It may well be that there are multiple different receptors for different EPEC strains. The animal studies in that case are particularly relevant to investigation of this hypothesis.

If there is an association with disease either in the infants or in the mouse model, character-ization of the red cell receptor will be important to our understanding host susceptibility. Characterization may be required for EPEC other than E2348/69 if the infant studies suggest that it would be useful. The inhibition of hemolysis by simple carbohydrates and enzyme treatments will be analyzed by ANOVA. The agglutination with commercial sera will be related to contact hemolysis by ANOVA or Kruskall Wallis depending on whether the data are normally distributed or not. If we are unable to confirm the carbohydrate hypothesis, we will evaluate other factors that might block bacteria-induced erythrocyte contact (collagen, gelatin, fibrinectin, fetuin, asialofetuin, laminin).(115)

Since it is already known that there are large differences in susceptibility to *Citrobacter rodentium* in different strains of mice (116) , we should be able to test the hypothesis that erythrocyte receptors reflect gut receptors and susceptibility. We anticipate that carbohydrate receptors are likely to be involved. Although the mouse receptors are unlikely to be related to the human receptors, these studies will address the concept that the erythrocyte receptors are related to the gut receptors for EPEC/Citrobacter. The mouse model data will be analyzed by linear regression relating hemolytic activity to days colonized and to colonic weight.

Specific Aim 3: Characterize the mechanism of human lactoferrin protection against EPEC infection and disease.

We hypothesize that the impairment in bacterial virulence that we have begun to define in *Shigella* has a parallel in EPEC and other needle complex producing enteropathogens. We have found that recombinant lactoferrin blocks EPEC E2348/69-mediated contact hemolysis. We hypothesize that the mechanism is disruption of the needle complex-multimeric EspA organelle. The effect of lactoferrin on the construction and function of the EspA organelle in vitro will be defined. Construction of the organelle will be monitored by scanning electron microscopy. The relationship between cell associated and secreted virulence antigens will be studied by EIAs and Western blots. The mechanism of the lactoferrin contact hemolysis blockade will be assessed by using lactoferrin that has been modified by site directed mutagenesis. Mutant lactoferrins will be made and expressed in a baculovirus system so that we can determine whether the mutations in the regions with similarity to the secretory proteins abrogates lactoferrin's effect in the contact hemolysis assay. The sites chosen for mutagenesis will be based on BLAST searches that have shown lactoferrin homology with type III secretory system proteins. An alternate approach will be to prepare and study peptides which based on the x ray crystallography data, represent surface exposed portions of the lactoferrin molecule. Site directed mutagenesis and lactoferrin purification will be done in collaboration with the Molecular Biology Core of Dr. Jiang. The EPEC needle (EscF) and outer ring (EscC) will be his-tag to

facilitate purification so that it can be determined whether they bind directly to lactoferrin and to mutated versions of lactoferrin that block or do not block contact hemolysis. Lactoferrin binding to EspA, EscF and EscC will be evaluated in Western blot, EIA, and immuno-precipitation (with anti-lactoferrin) experiments. Lactoferrin-sepharose affinity chromatography will be used to determine whether proteins in a lysate of EPEC containing protease inhibitors attach to lactoferrin. Sizing by SDS PAGE and N terminal sequencing will be used to determine whether eluted proteins are consistent with EscF or EscC or other type III secretory system proteins. Electron microscopy of needle complex reacted with lactoferrin and anti-lactoferrin or anti-EspA followed by immunogold staining will be used to further localize the site of lactoferrin interaction.

Anticipated results, data analysis, and alternate approaches: Our approaches for addressing the hypothesis that lactoferrin interferes with structure/function of the needle complex will be addressed morphologically by electron microscopy, genetically with mutated lactoferrins, physiologically by addressing the relationship between cell-associated and secreted virulence proteins, and biochemically by protein binding/affinity column and N terminal sequencing. It is anticipated that these multiple approaches will yield insights into the mechanism by which lactoferrin interferes with needle complex dependent events. Supernatant and cell associated proteins will be defined in the expectation that if lactoferrin blocks the formation of the needle complex, intracellular levels of the proteins that are normally secreted may rise while the amounts secreted fall. Differences in secretion will be defined by Student t test. We expect scanning electron microscopy to show loss or blunting of the needle complex-multimeric EspA organelle. The electron microscopy will be done as a confirmatory study to visually define the changes we hypothesize occur. The site directed mutagenesis contact hemolysis data on multiple mutant lactoferrins will be analyzed by ANOVA. If expression in the baculovirus system proves difficult, we will evaluate expression in a yeast system. The binding of lactoferrin/mutant lactoferrins to EscF and EscC in EIAs will be analyzed by ANOVA. Western blot studies in which denatured (SDS-PAGE) separated EPEC proteins as well as Westerns using non denatured native antigens will be done. The separated antigens will be transferred to nitrocellulose, blocked, and reacted with lactoferrin followed by peroxidase conjugated anti-lactoferrin; these studies may help determine whether lactoferrin reacts with one or many EPEC components during the induction of the needle complex/EspA organelle. The molecular weights of other components recognized may suggest other proteins worth investigation. It is possible that this approach will not work because antigens are denatured prior to transfer and may not reassume native conformations. To explore the possibility that lactoferrin interactions with LPS are responsible for inhibition of contact hemolysis, we will study cationic N terminal peptides to determine whether the regions of the molecule thought to be responsible for lipid A binding are responsible for inhibition of initial contact. These peptides have been kindly provided to us by Agennix Corp.

Specific Aim 4: Determine the relative contribution of human milk factors, including anti-EspA sIgA, lactoferrin, and oligosaccharides in protection from EPEC in tissue culture and animal models.

The purpose of these studies will be to define the relative importance of major protective factors and their interactions.

In vitro assessment of synergy: FAS and contact hemolysis assays will be used to determine whether the protective factors are additive or synergistic in their action. Affinity purified human sIgA to EspA, recombinant human lactoferrin, and protective oligosaccharides will be tested alone and in combination for ability to block FAS and hemolysis. Initial studies will use EPEC E2348/69; subsequent studies will use other clinical isolates from Mexico.

Citrobacter rodentium mouse model studies: The strain of mice used will be based on the studies of susceptibility outlined in aim 2. In each set of experiments the following information will be collected: daily body weight; fecal samples for culture and antibody measurement; blood collected at sacrifice for antibodies; colon analyzed at necropsy for weight and histology. Two types of mouse experiment are anticipated.

First, for synergy studies comparing the effect of protective factors in combination, mice will be weaned and then fed either affinity purified human anti-EspA sIgA, recombinant human lactoferrin, and/or oligosaccharide prior to and in the days after challenge with *C. rodentium.* For these studies, we will add protective factors to the pups water (with 50mM phosphate buffered saline and 0.1% bovine serum albumin to stabilize proteins). Each factor will be studied alone first to determine the effective dose range. Subsequently each factor will be studied at a subeffective dose to determine whether synergy occurs with the other factors. An irrelevant affinity purified sIgA [anti-LPS of *S. flexneri* serotype 5], protein [ovalbumin], or sugar [lactose] will be fed to control animals. Pups will be infected with *C. rodentium* inoculum at 21 days (1 day after initiation of protective factor addition to their water) and cultured daily for the next ten days to determine effect of the putative protective factor on colonization. Animals will be sacrificed for colonic weight and pathology 10 days post infection. Pup feces will be evaluated initially and at sacrifice for presence of anti-EspA IgA to assure that the mice were not already immune or receiving protective antibody from their mothers and to assure that the dose of antibody chosen is adequate to have detectable levels in feces. (117) After sacrifice, a sample of blood will be removed for EIA analysis of antibodies to EspA and other *C. rodentium* antigens. Rechallenge studies will be done in groups of animals three months later (as described (31)) to determine whether active immunity has developed during feeding of antibody, oligosaccharide or lactoferrin. Groups of animals used in these studies will have been followed with daily cultures in the months after infection so that duration of excretion after infection can be determined and animals documented to be free of infection at the time of rechallenge.

Second, active immunity in mice will be studied to complement the passive immunity data above. Mice will be immunized with purified EspA to induce mucosal immunity beginning at one week of age. The method developed by Daynes will be used to induce mucosal immunity. (118) Mice will be immunized intradermally with 1ug of EspA and 0.1ug choleratoxin in 5uL of aluminum hydroxide (275ug/mL). Sham immunized animals will receive either choleratoxin or choleratoxin plus ovalbumin. Peak serum and secretory responses occur at 14-21 days using this protocol. At 21 days old pups will be weaned and challenged with *C. rodentium.* The duration of fecal excretion and daily body weight will be assessed during the subsequent three months in immunized and sham immunized animals. Fecal samples will be collected to determine the presence, timing of development, and amount of antibodies to virulence antigens. Serum will be collected at sacrifice for determination of antibodies to virulence antigens. In a set of experiments animals will be sacrificed at ten days to study the proliferative changes in the distal colon that occur by that point. (119, 120) Colonic weight (121) and morphology (117) will be assessed for evidence of colonic hyperplasia. Groups of immunized and sham immunized animals will be studied at three months post immunization to determine the duration of mucosal immunity. Animals will be challenged with *C. rodentium* and monitored for excretion, daily weight, and pathology over the subsequent ten days. If protection is demonstrated, longer-term studies will be evaluated.

Anticipated results, data analysis, and alternate approaches: To address the hypothesis that the protective factors work synergistically, we are using both in vitro model systems and in vivo mouse approaches. We predict that the protective factors may be additive or synergistic; each is likely to act on the needle complex/EspA organelle. The relative importance of each protective factor will be defined by these in vitro assays and confirmed in the mouse model studies described below. The FAS in HEp2 cells will be read by a blinded observer to minimize bias; significance of differences for contact hemolysis and FAS will be determined by ANOVA. We anticipate that protective mucosal immunity will be induced by active immunization with EspA and that this immunity will be persistent. Vaccine efficacy with 95% confidence intervals will be evaluated. For the studies of factors in combination we anticipate using subinhibitory concentrations of each factor and determining outcome as indicated by ANOVA of colonic weights and duration of excretion. The role of each factor alone and in combination in promoting long term active immunity will be defined in the rechallenge studies. We predict that protection during first exposure will allow development of active protective immunity. This is conceptually important because it has never been shown that exposure/colonization occurring under the protection of milk factors leads to development of active immunity. The studies using oligosaccharide may not work because the receptor in mouse gut for *C. rodentium* may not be relevant to the human EPEC receptor(s) and the related milk oligosaccharide(s); however, the in vitro studies may adequately clarify their role.

For calculation of number of animals required we have assumed that 90% of animals will become ill on challenge with *C. rodentium* (in one study 48 of 48 became ill.) (120) Assuming a reduction in frequency of colonic hyperplasia to 25% is likely if IgA, lactoferrin, or oligo-saccharide is protective in the pups, we should be able to show a decrease from 90% to 25% ill with a power of 0.80 and alpha 0.01 with only 15 animals per group for each condition studied. Significance of differences will be determined by contingency table analysis. Alpha has been set at 0.01 because of the multiple comparison design. Thus, for each factor studied, we expect to be able to determine the ability of passively acquired factor to protect with approximately 15 treated pups and 15 sham treated pups. Given that each factor may be more effective than estimated, it is likely that even smaller numbers of animals will be required. If on the other hand the differences are less than expected, we will need to base new estimates on the observed frequencies and reallocate resources to do the studies. Significance of differences will be determined by contingency table analysis. Further details regarding animal use are discussed below in "Section F. Vertebrate animals." There are other animal models of EPEC infection. The advantage of using mice is reproducibility of the model, cost, and ease of handling/housing small animals. We believe that the mouse model has significant enough advantages that we favor its use.

Specific Aim 5: Determine the relative contribution of human milk factors, including anti-EspA antibodies, lactoferrin, and oligosaccharides in protection from EPEC in breastfed children.

In the current cohort approximately 7% of diarrhea episodes are associated with isolation of an EPEC. The role of milk in protection from illness during *first EPEC infection* of breastfed infants will be determined using the 1998-2003 cohort. As described in the Core, EPEC infection has been defined from weekly stool cultures obtained for each infant and stool cultures during each diarrheal illness during the first two years of life. Over the last 15 years approximately 20% of episodes of diarrhea in Mexico have two or more organisms defined. Symptomatic children who have evidence of mixed infection with rotavirus, ETEC, EIEC, STEC, *Campylobacter, Salmonella, Shigella*, calicivirus, enteric adenovirus, astrovirus, giardia, cryptosporidium, or *Entamoeba histolytica* will be excluded from further consideration because it would not be possible to know whether symptoms were due to the second pathogen. The virulence genes of EPEC isolates from symptomatic and asymptomatic infections will be characterized and their subtypes defined. The children who have gastroenteritis with EPEC *only*, as well as the asymptomatic first EPEC infections, will have their *E. coli* characterized for virulence by FAS (122) in a HEp2 culture system, which will allow us to ensure that EPEC isolates from asymptomatic children remain pathogenic. Some *eae*+ organisms may be FAS-negative and not fully virulent. (123) Therefore, any child found to have an EPEC with impaired virulence as defined by FAS, will be excluded from subsequent analysis. An *E. coli* that is *eae+stx-* will, for purposes of this study, be considered an EPEC since *eae+stx- E. coli* have been associated with pediatric diarrhea even when they do not belong to the classic serogroups.(124)

In the current cohort, the incidence of symptomatic EPEC infections is 0.15 episodes per child-year in breastfed infants (n=306). To address this specific aim, we will begin with a nested case-control study of anti-EspA milk sIgA protection using milk samples collected just prior to symptomatic and asymptomatic infections in breastfed infants. Based on detection rates through 2001, we estimate detection of at least 37 symptomatic first EPEC infections in breastfed children; of which 30 (80%) are free of co-infection with any other pathogens We also anticipate at least 60 asymptomatic EPEC infections. This sample size provides >90% power to detect expected differences in maternal milk anti-EspA antibody in children with symptomatic (n=30) vs. asymptomatic (n=60) first EPEC infections, and is sufficient (>80% power) to support four secondary comparisons of maternal milk antibody protection against symptomatic infection. After completion of the nested case-control study of sIgA protection, we will conduct a longitudinal study to examine the interrelationships and protective effects of all three milk factors (anti-EspA sIgA, oligosaccharide and lactoferrin) to protect against EPEC diarrhea in breastfed children. We will measure milk for titer of anti-EspA by EIA, the concentraton of lactoferrin by EIA, and presence or absence of the oligosaccharide defined in aim 1 by HPLC in Dr. Newburg's laboratory. All three factors will be analyzed in the milk of all study mothers in a sample collected at 4-6 weeks postpartum. Analysis of the entire cohort will provide us with >80% power to detect a 2.5-fold difference in the incidence of symptomatic EPEC

infection between high and low levels of each individual protective milk factor - sIgA, lactoferrin and oligosaccharide concentration – allowing for a potential correlation of 0.4 among these factors. We will analyze these data using Poisson regression to investigate the relative contribution of each of these factors to protect against symptomatic EPEC infection

Anticipated results, data analysis, and alternate approaches: Justification for conducting both nested case-control and longitudinal study designs is as follows. The concentration of antibody in human milk varies in response to the mother's environmental exposure. It is our experience that analysis of milk antibody in samples collected just prior to infection best represents the true protective exposure of the child. Thus, to measure the protective effect of secretory antibody as the primary independent variable, we use a nested case-control design, accounting for the milk concentrations of oligosaccharide and lactoferrin as co-variates. On the other hand, change in milk oligosaccharide occurs systematically over the course of lactation in response to genetic control rather than maternal exposure. Further, our data suggest that the association between milk oligosaccharide and risk of diarrhea is best studied using a standardized sample timing, e.g., at 4-6 weeks postpartum. Thus, to measure the protective effect of oligosaccharide and lactoferrin, we need to use the longitudinal design, accounting for milk secretory antibody as a covariate. In all study children, the milk sample at 4-6 weeks will be assayed for all three milk factors – sIgA, oligosaccharide and lactoferrin. In children infected with EPEC while breast-feeding (approximately n=90) the milk sample collected just prior to infection will also be assayed for all three milk factors. In subjects with samples assayed from both time points, we will examine the correlation in values within and between the two milk samples.

We predict that antibody in the milk sample collected just prior to infection is the most important protective factors. However it is possible that either of the other factors could be just as potent. We will therefore analyze the patients with the lowest amounts of each protective factor and relate symptom status to the concentrations of the other factors. Although presence of anti-EspA could simply be a reflection of anti-EPEC immunity involving multiple antigens, the low frequency and low levels of antibodies to other antigens (125) make it likely that anti-EspA is the dominant antibody. Although protection may be due to the combined effect of several antibodies, our focus is on definition of the most important, most consistently produced antibodies.

If unexpectedly, we find that neither anti-EspA, lactoferrin, nor oligosaccharide alone or in combination is protective, we will consider other potentially important antibodies [anti-intimin, anti-BFP, anti-O antigen, anti-H antigen]. If these studies are negative, we will expand the analysis to include anti-EspB, anti-EspD, anti-EspC, anti-EspF, anti-Tir, and other antigens as they are described in the literature. Some of these other virulence proteins are immunogenic, but are very poor candidates for protective immunity. EspB, EspD, EspF, and Tir are secreted and elicit antibodies. However, it is not likely that such antibodies would be protective if, as current data suggest, these antigens are injected directly into host cells via the EspA organelle. We doubt that antibodies to BFP will be important based on recent observations in human intestinal organ culture that suggest that bundlin may not be involved in initial adherence although it may have a role in interactions between bacteria. (126) However, since BFP appears to be an important virulence factor in adult volunteers, the question remains open.(127) Likewise, the low frequency of antibodies to many of the O antigens, makes them poor candidates to explain milk protection. Nevertheless, if none of the putative protective factors relates to infant well being, titer will also be determined against the O and H antigens of the EPEC serotype isolated from the child.

METHODS

Detection of EPEC and characterization of virulence genes/phenotypes. Pools of five fecal *E. coli* are screened by an N terminal specific *eae* PCR. The primers [5'-CTGAACGGCGATTACG CGAA-3' and 5'-CCAGACGATACGATCCAG-3'] amplify a 917bp segment encoding a portion of the conserved N terminal region. (128) Positive colonies will then be defined by PCR on each individual *E. coli* from a positive pool. O and H serotyping will be done in Dr. Ruiz Palacios lab using Difco reagents; a random set of organisms will have serotype confirmed in a reference laboratory. We will define the variants of *espA*, *espB*, and *tir* by

PCR and RLFP as described by China et al.(129) The variants of virulence genes will be characterized. For *espA* a multi-plex PCR is done using a mix of the following primers and degenerate primers: (forward) [5'-TGAGGCATCTAARGMGTC-3'] (R=A+G, M=A+C), (reverse) [5'-ATCACGAATACCAGTTAC CA-3'], (reverse) [5'-GCTGGCTATTATTGACCG-3'], and (reverse) [5'-TGCCTTTCTTATTCTT GTCA-3']. PCR is done as described by China. The products are 269bp for *espA*α 101bp for *espA*β and 172bp for *espA*γ. Restriction enzyme digestion yields products of 189 and 80 bp for *espA*α with *HaeII*, 50 and 61bp for *espA*β with *FokI*, and 64 and 108 bp for *espA*γ with *MnlI*. For *espB* a multiplex PCR is done using the primers: (forward) [5'-GCCGTTTTTGAGAGCCA-3'], (reverse) [5'-CTTTCCGTTGCCTTAGT-3'], (reverse) [5'-GCACCAGCAGCCTTTGA-3'], and (reverse) [5'-TCCCCAGGACAGATGAGAT-3']. PCR is done as described by China. The products are 94bp for *espB*α, 233bp for *espB*β, and 188bp for *espB*γ. Restriction enzyme digestion of *espB*α yields products of 50 and 44bp with *HpaII*, and either 133 and 100bp for *espB*β or 94 with *espB*γ with *Sau3A*. For *tir* a multiplex PCR is done using the primers: (forward) [5'-CRCCKCCAYTACCTTCACA-3'] (R=A+G, K=T+G, Y=C+T), (reverse) [5'-GATTTT TCCCTCGCCACTA-3'], B141 (reverse) [5'-GTCGGGCAGTTTCAGTTTCAC-3'], and (reverse) [5'-CGCTAACCTCCAAAC CATT-3'] to amplify products of 342bp for *tir*α 781bp for *tir*β and 560 bp for *tir*γ. PCR conditions are as described by China. Restriction enzyme digestion of these amplicons yields fragments of 127 and 215bp with *PstI* for *tir*α 240 and 320bp with *XhoI* for *tir*β and 321 and 460 with *EcoRV* for *tir*γ. The variants of intimin are defined by PCR as described by Oswald et al. (29) A multiplex PCR with *PstI* digestions and RFLP analysis yields 7 patterns α1, α2, β1, β2, γ1, γ2, and ε. The forward primer is [5'-CCCGAATTCGGCACAA GCATA AGC-3'], and the reverse primers [5'-CCCGGATCCGTCTCGCCAGTATTCG-3'], [5'-CCCGAATTCTTATTTTACACAAGTGGC-3'], [5'-CCCGAATTCTTATTCTACACAAACCGC-3'], [5'-CCCGTGATACCAGTAC CAATTACGGTC-3'], and [5'-AGCTCACTCGTAGATGACGGCAA GCG-3']. Bundlin variability will be defined by sequencing PCR products of the *bfpA* genes as described by Blank et al.(130) The forward primers to be used are as described by Donnenberg -#6 [5'-TCTTGGTGCTTGCGTGTC-3'], #28 [5'CGCGGATCCATGGTTTCTAAAATCATGAAT-3'], #362 [5'AGGTCTGTCTTTGATTGAA-3'], #423 [5'-G ATTATTCCGTGACCTATT-3'], and the reverse primers are #29 [5'-GCGAAGCTTTTACTTCATAAAATATGTAAC-3'], #363 [5'-CCTGA GTAAAACAGGAT AC-3'], and #382 [5'-TCCTTCGGGTGATTGTGTA-3']. The primer pairs used are 28 and 29, 362 and 363, 423 and 363, 6 and 382, 423 and 382. If the amplified product is the expected size it is purified using Wizard PCR Preps DNA Purification System (Promega). If additional unwanted amplicons are present preparative agarose gel electrophoresis is done. Sequencing will be done by in the medical school core facility. The PCR conditions will need to be worked out since they are not defined in Blank's paper. Designation of type based on sequencing (α1, α2, α3, β1, β2, β3, β4, β5) will be done as described by Blank et al.(130) Given the limited sequence data currently in Genbank for EspC, EspD and EspP, we are not currently planning to look for variants of these virulence antigens; we will however modify this plan if additional data are published describing variants.

Preparation of Antigens for EIA and immunization protocols. LPS is extracted in our lab with phenol water using the method described by Westphal and Jann (131) or purchased (Sigma) having been prepared by the same method. H antigens will be prepared as described by Ibrahim (132) . For preparation of histidine tagged proteins we are using Invitrogen constructs. *Taq* polymerase PCR products for the desired virulence protein are cloned in frame into pCRT7/NT-TOPO linearized vector and cloned into chemically competent TOP10F' cells [F'{*lacI*^{q}, Tn*10* (Tet^R)} *mcrA*Δ (*mrr-hsd* RMS-*mrcBC*) ϕ80*lacZ* ΔM15 Δ*lac*X74*recA*1*deoR araD*139Δ (*ara-leu*) 7697 *galU galK rspL* (Str^R)*endA*1*nupG*] for stable propagation and maintenance of recombinants. This vector has a phage T7 promoter to get high level expression, an N-terminal 6xHis for purification on a nickel affinity column, and an enterokinase cleavage site to allow purification of His free protein. After demonstration of orientation and sequencing, the recombinant plasmid is inserted into BL21(DE3) [F- *ompT hsdS*$_B$ (r_B-m_B-) *gal dcm* (DE3)] for expression; if the products are toxic to *E. coli* the plasmid is expressed in BL21(DE3)pLysS [F- *ompT hsdS*$_B$ (r_B-m_B-) *gal dcm* (DE3) pLysS (Cam^R)] and expression induced by IPTG. We will also investigate use of the Echo Cloning system for making 6xHis fusion tags. This system allows PCR products to cloned into pUni/V5-His-TOPO and recombined into an expression vector by Cre recombinase mediated fusion. Multiple vectors are available for

expression using this system. The vectors for expression in *E. coli* include pBAD/Thio-E for tightly regulated expression, pCRT7-E for high-level inducible expression, and pRSET-E for expression with an N terminal tag. Detection of the expressed fusion vector is simplified because of the presence of the V5 epitope. The availability of PIR1 and PIR2 strains for propagation of vectors allows even toxic products to be expressed. If some of the peptides cannot be isolated using these approaches, we will make fusion proteins with either 6Xhis-DHFR, maltose binding protein, or glutathione S-transferase protein so that affinity purification of the target peptides/proteins will be possible. *E. coli* M15 with the plasmids encoding either EspA or EspB cloned from O26:H- strain 413/89-1 and tagged with six histidines at the C-terminus is used to purify his-tagged EspA and EspB. Purification is done under non-denaturing conditions on nickel nitrilotriacetic acid coupled to Sepharose CL-6B metal affinity chrom-atography with imidazole elution.

Affinity purification of anti-EspA sIgA. An affinity column is prepared by linking his-tagged EspAβ from O26:H- strain 413/89-1 to CNBr-activated sepharose 4B. Pooled aqueous fraction of breast milk is applied to the column. Elution of sIgA is done using 100 mM glycine-HCl pH 2.5. Fractions are collected in tubes containing 1 M TRIS pH 8 and BSA for a final concen-tration of 0.1% BSA. Eluted sIgA is dialyzed against PBS pH 7.4, sterile-filtered and stored at –20°C. EIA and Western blot are done to show specificity of the purified sIgA against EspA. Purified sIgA is diluted to 1.5×10^{-9}M for in vitro assays.

Other reagents. Oligosaccharides will be purified as described in the Glycobiology Core of Dr. Newburg. Recombinant human lactoferrin has been provided by Agennix Corp. We currently have on hand 50gm of purified lactoferrin; this is enough for all of the anticipated studies. Human lactoferrin (Sigma) will be used for standard curves in lactoferrin EIA. Frank Ebel has kindly provided monoclonal antibodies to EspA, EspB, EspC, EspD, and EspE. Site directed mutagenesis on the lactoferrin cDNA will be done by the Molecular Biology Core of Dr. Jiang in Cincinnati with testing in model systems in Houston. Dr. Jiang will use the baculovirus expression system for these studies since induction of high level expression of lactoferrin in *E. coli* is likely to be lethal.

Hep2 cell FAS assay. Bacteria are grown overnight in Luria Bertani broth at 37°C without shaking. Overnight-grown bacteria is inoculated into 50 mL of DMEM with 25mM HEPES, incubated at 37°C, 5% CO_2. Bacterial concentration is estimated by turbidity and diluted to the appropriate concentration with DMEM-HEPES prior to infecting Hep2 cells. Eight-chamber slides are seeded with HEp-2 cells and grown overnight to obtain a subconfluent monolayer. HEp-2 cell plates are inoculated with bacteria and incubated at 37°, 5% CO_2. After 1 hour, cell media is replaced with gentamicin-containing media (100 μg/mL of gentamicin). After 6 hours of total incubation time to allow maximum actin polymerization, cells are washed three times, fixed, and permeabilized. Cells are stained with BODIPY-phallacidin to visualize actin and DAPI to visualize bacterial and HEp-2 cell DNA. HEp-2 cells with ≥ 3 bacteria adherent associated with actin polymerization are considered positive. The proportion of cells showing characteristic fluorescent actin staining is determined by counting at least 500 cells. For experiments using antigen specific affinity purified sIgA either the affinity purified anti-EspA sIgA or an irrelevant affinity purified antibody [anti-*S. flexneri* 5 LPS] as control is added to obtain a final bacterial concentration of 1:50 HEp-2 cell/bacteria ratio. This is incubated for 1 hour 37°C, 5% CO_2. After washing twice, Hep2 cells are inoculated with pre-incubated bacteria, incubated for one hour, media replaced with gentamicin containing media, incubated an additional 6 hours and stained for actin as above.

Hemolysis assay. Human red blood cells are collected in ACD and stored at 4C until use. An aliquot is removed prior to assay, washed x3 with PBS and diluted in DMEM HEPES to a final concentration of 2%. The erythrocytes are then incubated with approximately 10^8 EPEC in DMEM HEPES at 37C for 6 hrs. At the end of the incubation the cells are pelleted and the supernatant assayed for released hemoglobin (543 nm).

***C. rod ntium* challenge studies.** Pups will be infected with *C. rodentium* (*C. freundii* biotype 4280) inoculum in a dose reported to cause illness in nearly 100% of animals (approximately 1.5×10^7) and sacrificed 10 days later. Colonic weight (121) and morphology (117) will be assessed to determine presence of colonic

hyperplasia. Rechallenge studies will use a larger inoculum as previously described.(31) Culture of *C. rodentium* is facilitated by the nalidixic acid resistance of the organism. Fecal samples will be plated on MacConkey media with nalidixic acid incorporated into the medium.

Overview of experimental design for mouse studies

Experimental protocol #1: evaluation of strain variability in susceptibility
Day 1—21 day old pups of multiple inbred strains [A/J, BALB/c, DBA/2, SJL/J, FVB, CBA/J, C57BL/6, and C3H] will be inoculated with *C. rodentium* (approximately 1.5×10^7) by orogastric tube (n=15/group=120 mice). Animals will be kept in isolator cages.
Day 2-30 –animals will be weighed daily, observed for signs of illness, and fecal cultures obtained.
Day 30—animals will be sacrificed and blood collected for contact hemolytic assay. Colons will be removed, washed, weighed, and examined histologically.
After this experiment all subsequent studies will be done with a single strain of mice.

Experimental protocol #2: Immunization with EspA to determine effect on duration of carriage and well being [strain of mice determined by experimental protocol #1 above]
Day 1: birth of pups
Day 7: immunization with either:

- Group1: EspA (1ug subcut.) alone
- Group 2: EspA (1ug) with choleratoxin as an adjuvant [0.1ug] subcut.
- Group 3: ovalbumin 1ug subcut.
- Group 4: ovalbumin 1ug plus choleratoxin as an adjuvant [0.1ug] subcut.
- Group 5: immunized with vehicle alone

There will be 15 mice per group [5x15=75 mice]. Mice will be immunized intradermally in the foot pad. The proteins will be delivered in 5uL of aluminum hydroxide (275ug/mL).
Day 20: feces will be cultured from each animal to assure that they are culture negative for *C. rodentium.*
Day 21: animals will be inoculated with *C. rodentium* (approximately 1.5×10^7) by orogastric tube.

Individual animals will be kept in isolator cages from this point.
Day 22-51: stool samples will be cultured and evaluated for anti-EspA antibody; animals will be weighed and observed for signs of illness.
Day 51: animals will be sacrificed so that blood may be obtained, colons weighed and histologically examined.

Experimental protocol #3: Immunization with EspA to determine effect on pathology [strain of mice determined by experimental protocol #1 above]
Day 1: birth of pups
Day 7: immunization with either:

Group1: EspA (1ug subcut.) alone
Group 2: EspA (1ug) with choleratoxin as an adjuvant [0.1ug] subcut.
Group 3: ovalbumin 1ug subcut.
Group 4: ovalbumin 1ug plus choleratoxin as an adjuvant [0.1ug] subcut.
Group 5: immunized with vehicle alone

There will be 15 mice per group [5x15=75 mice]. Mice will be immunized intradermally in the foot pad. The proteins will be delivered in 5uL of aluminum hydroxide (275ug/mL).
Day 20: feces will be cultured from each animal to assure that they are culture negative for *C. rodentium.*
Day 21: animals will be inoculated with *C. rodentium* (approximately 1.5×10^7) by orogastric tube.
Individual animals will be kept in isolator cages from this point.
Day 22--32: stool samples will be cultured and evaluated for anti-EspA antibody; animals will be weighed and observed for signs of illness.
Day 32: animals will be sacrificed so that blood may be obtained, colons weighed and histologically examined.

Experimental protocol #4: Immunization with EspA with challenge at 90 days to determine duration of mucosal immunity
Day 1: birth of pups
Day 7: immunization with either:

Group1: EspA (1ug subcut.) alone
Group 2: EspA (1ug) with choleratoxin as an adjuvant [0.1ug] subcut.
Group 3: ovalbumin 1ug subcut.
Group 4: ovalbumin 1ug plus choleratoxin as an adjuvant [0.1ug] subcut.
Group 5: immunized with vehicle alone

There will be 15 mice per group [5x15=75 mice]. Mice will be immunized intradermally in the foot pad. The proteins will be delivered in 5uL of aluminum hydroxide (275ug/mL).
Day 90: feces will be cultured from each animal to assure that they are culture negative for *C. rodentium.*
Day 91: animals will be inoculated with *C. rodentium* (approximately 8×10^8) by orogastric tube. Individual animals will be kept in isolator cages from this point.

Day 92--102: stool samples will be cultured and evaluated for anti-EspA antibody; animals will be weighed and observed for signs of illness.
Day 102: animals will be sacrificed so that blood may be obtained, colons weighed and histologically examined.

Experimental protocol #5: Passive antibody protection by affinity purified anti-EspA –dose response curve [strain of mice determined by experimental protocol #1 above]
Day 1: birth of pups
Day 20: Daily administration of anti-EspA in buffer or buffer alone in water begins. One group of mice will receive an affinity purified sIgA to an irrelevant antigen (*S. flexneri* 5 LPS) at the same concentration of sIgA as group 2.

Group 1: buffer alone
Group 2: anti-EspA in buffer (at the concentration found in human milk)

Group 3: anti-EspA at one tenth the concentration in human milk
Group 4: anti-EspA at one hundreth of the concentration in human milk
Group 5: anti-flexneri LPS at same concentration as antibody in group 2
There will be 15 mice per group [5x15=75 mice].
Day 21: animals will be inoculated with *C. rodentium* (approximately 1.5×10^7) by orogastric tube. Daily administration of buffer or antibody in buffer will continue. Individual animals will be kept in isolator cages from this point.
Day 22-32: stool samples will be cultured and evaluated for anti-EspA antibody; animals will be weighed and observed for signs of illness.
Day 32: animals will be sacrificed so that blood may be obtained, colons weighed and histologically examined.

Experimental protocol #6: Assessment of ability of recombinant human lactoferrin to protect mice from *C. rodentium.*
Day 1: birth of pups
Day 20: Daily administration of lactoferrin in buffer or buffer alone in water begins
Group 1: buffer alone
Group 2: lactoferrin in buffer at the concentration found in human colostrum [10mg/mL]
Group 3: lactoferrin at 1mg/mL
Group 4: lactoferrin at 0.1mg/mL
Group 5: ovalbumin at 10mg/mL.
There will be 15 mice per group [5x15=75 mice].
Day 21: animals will be inoculated with *C. rodentium* (approximately 1.5×10^7) by orogastric tube. Daily administration of buffer or lactoferrin in buffer will continue. Individual animals will be kept in isolator cages from this point.
Day 22-32: stool samples will be cultured and evaluated for anti-EspA antibody; animals will be weighed and observed for signs of illness.
Day 32: animals will be sacrificed so that blood may be obtained, colons weighed and histologically examined.

Experimental protocol #7: Assessment of ability of milk oligosaccharide to protect mice from *C. rodentium.*
Day 1: birth of pups
Day 20: Daily administration of oligosaccharide in buffer or buffer alone begins
Group 1: buffer alone
Group 2: oligosaccharide in buffer at the concentration found in human colostrum,
Group 3: oligosaccharide at a dose ten fold lower than group 2
Group 4: oligosaccharide at a dose one hundred fold lower than group 2
Group 5: lactose at the same concentration as the oligosaccharide in group 2
There will be 15 mice per group [5x15=75 mice].
Day 21: animals will be inoculated with *C. rodentium* (approximately 1.5×10^7) by orogastric tube. Daily administration of buffer or oligosaccharide in buffer will continue. Individual animals will be kept in isolator cages from this point.
Day 22-32: stool samples will be cultured and evaluated for anti-EspA antibody; animals will be weighed and observed for signs of illness.
Day 32: animals will be sacrificed so that blood may be obtained, colons weighed and histologically examined.

Experimental protocol #8: Assessment of ability of combinations of anti-EspA, recombinant human lactoferrin, and/or milk oligosaccharide to synergistically protect mice from *C. rodentium.*
Day 1: birth of pups
Day 20: Daily administration begins of one protective factor at subtherapeutic dose with testing of a second factor over a range of concentrations from the low end of therapeutic to fractions of that concentration
Group 1: buffer alone

Group 2: subtherapeutic concentrations of factor 1
Group 3: subtherapeutic concentration of factor 1 with a dose of the factor 2 equivalent to the lowest concentration found to be protective in the above experiments [protocols 5-7]
Group 4: subtherapeutic concentration of factor 1 with a dose of the factor 2 equivalent to one half of the concentration used in group 3
Group 5: subtherapeutic concentration of factor 1 with a dose of the factor 2 equivalent to one quarter of the concentration used in group 3
There will be 15 mice per group [5x15=75 mice].
Day 21: animals will be inoculated with *C. rodentium* (approximately 1.5×10^7) by orogastric tube. Daily administration of buffer or protective factors in buffer will continue. Individual animals will be kept in isolator cages from this point.
Day 22-32: stool samples will be cultured and evaluated for anti-EspA antibody; animals will be weighed and observed for signs of illness.
Day 32: animals will be sacrificed so that blood may be obtained, colons weighed and histologically examined.

Experimental Protocol 9: eight additional variations of experiment #8 in which either two or three factors are combined as outlined to determine synergy. Each of these variations will require 75 mice. Therefore the total number of mice required will be 75x8=320

Experimental protocol #10: development of active immunity during passively acquired protection
Day 1: birth of pups
Day 20: feces will be cultured from each animal to assure that they are culture negative for *C. rodentium.*
Day 20-30: Daily administration begins of a protective factor at therapeutic dose (determined in experimental protocols 5-7)
Group 1: buffer alone
Group 2: protective factor in buffer
Group 3: protein, carbohydrate, or antibody control for group 2
There will be 15 x 3 =45 for each of the three groups of factors; n=135 total
Day 21: animals will be inoculated with *C. rodentium* (approximately 1.5×10^7) by orogastric tube. Individual animals will be kept in isolator cages from this point.
Day 22-90: animals will be cultured to determine duration of fecal carriage.
Day 91: animals will be inoculated with *C. rodentium* (approximately 8×10^8) by orogastric tube. Individual animals will be kept in isolator cages from this point.
Day 92--102: stool samples will be cultured and evaluated for anti-EspA antibody; animals will be weighed and observed for signs of illness.
Day 102: animals will be sacrificed so that blood may be obtained, colons weighed and histologically examined.

The total number of mice that we are currently anticipating utilizing for these studies is approximately 1130 over the next five years.

(2) Animals are justified for these studies because the issues addressed in the animal studies cannot be as clearly defined in humans without prohibitive expense. In mice it is possible to do challenge studies that can be better controlled than studies in humans. To answer the questions regarding protection, it is therefore necessary to study an animal model. There are a variety of animals (e.g. rabbits, pigs, cats, dogs, etc) that develop infection with EPEC. Of these, mice are the most practical. The model is highly reproducible and therefore relatively small numbers of animals are required. Illness occurs in a predictable time so that suffering can be minimized. No animal will be allowed to become moribund. Animals will be isolated to prevent spread of the infection to the mouse colony.

For calculation of number of animals required we have assumed that 90% of animals will become ill on challenge with *C. rodentium*. Assuming a reduction in frequency of colonic hyperplasia to 25% is likely if IgA, lactoferrin, or oligosaccharide is protective in the pups, we should be able to show a decrease from

90% to 25% ill with a power of 0.80 and alpha 0.01 with only 15 animals per group for each condition studied. Significance of differences will be determined by contingency table analysis. Alpha has been set at 0.01 because of the multiple comparison design.

Affinity purified antibodies to EspA will be used for passive immunization. Active immunization studies will use purified EspA, ovalbumin, and choleratoxin. Studies with lactoferrin will use recombinant material provided by Agennix Corp. Purified human milk oligosaccharides will be provided by Dr. Newburg.

(3) The veterinary facility of the University of Texas Medical School at Houston meets all NIH guidelines and is staffed by full time veterinarians. Review by the Institutional Animal Care and Use Committee of UTHSC is pending at the time of application.

(4) No procedures are anticipated that should cause more than very brief discomfort, distress, pain or injury. The organisms will be inoculated by orogastric tube without anesthesia; since the analogous procedure in humans (including children) is done without analgesia or anesthesia, we are not planning use of anesthesia or analgesia. In the protocol involving subcutaneous immunization, 5uL of vaccine will be administered into the foot pad subcutaneously. Our veterinarians have advised me against using analgesia for this procedure.

(5) Euthanasia will be done following the recommendations of the Panel on Euthanasia of the American Veterinary Medical Association. Carbon dioxide gas will be used.

Escherichia coli Stable Toxin (ST) Project

A. Specific Aims

Stable toxin (ST)-associated *Escherichia coli* is a major cause of diarrhea in many parts of the world. The incidence of ST-associated diarrhea is lower in breastfed than in formula-fed infants. This project is based on the hypothesis that a specific human milk fucosyloligosaccharide inhibits the binding of ST to its host receptor, thus preventing diarrhea. This investigation aims to define the structural determinants of this inhibition, their mechanisms of action, and the genetic control of their expression in human milk through the following aims:

1. Synthesize a complete combinatorial library of fucosylated trisaccharides and tetrasaccharides that encompass the possible ultimate products of the human milk fucosyltransferases of the Lewis and secretor gene family.
2. Test each of these trisaccharides and tetrasaccharides, individually and in combination, for their ability to inhibit ST-induced diarrhea in the suckling mouse and compare their activity with that of the human milk oligosaccharide fraction.
3. Investigate the mechanism of ST inhibition by synthetic and natural milk oligosaccharides in a human enterocyte cell culture system of T84 cells and in rat intestine.
4. Determine the genetic basis of variability in concentrations of the oligosaccharides in human milk that protect against ST.

Significance. These studies are designed to expand our understanding of the genetic basis for the expression of the ST-inhibitory fucosyloligosaccharide, the relative protection it affords breastfeeding infants against ST-associated diarrhea, the structural determinants of this protection, and the relative efficacy of various forms of the structural determinants. The findings of this research will provide additional support for breastfeeding promotion, clarify the genetic diversity of these factors, and enable supplements to be developed to protect children and adults at risk for ST-associated diarrhea.

B. Background and Significance

Human cost of diarrheal disease. Infectious diarrhea is responsible for more than five million deaths annually (1). Most of this mortality occurs in developing countries among children under 5 years of age, in whom it is a leading cause of death. Three million of these children die each year from dehydration combined with malnutrition (2,3). In addition to the cost in human life, diarrhea also burdens the economies of developing countries; in northeast Brazil, for example, diarrhea may account for 25% of total health care costs (4). Diarrheal disease is an important health problem in developed countries as well (5). In the United States, over 200,000 children under five years of age are hospitalized each year with acute diarrheal disease, accounting for nearly 880,000 inpatient days, over 500 deaths, and almost one billion dollars of inpatient cost per year (6). Very-low-birth-weight infants are at 100 times greater risk for mortality due to diarrhea than are other infants (7). Enterotoxin-producing *Escherichia coli* (ETEC) are common causes of infantile diarrhea, having been identified in 14%–19% of all cases of diarrheal infections in Bangladesh and Indonesia; of these cases, the majority were positive for heat-stable toxin (ST) (8,9). In a study of Argentine children with ETEC-associated diarrhea, only ETEC strains that produce ST were associated directly with diarrhea (P=0.0035) (10). In addition, ETEC diarrheal outbreaks have been reported, but are underestimated due to difficulties in laboratory identification of this pathogen (11).

Stable toxin of *E. coli* (ST). ETEC produces two types of enterotoxins, labile toxin and stable toxin (ST). The labile toxin is similar to cholera toxin and the toxin of *Campylobacter jejuni*. The heat-stable enterotoxins are a family of small cysteine-rich peptides produced by some *E. coli* strains and other enteric bacteria including *Yersinia enterocolitica*, non-cholera *Vibrio*, and *Citrobacter* spp. Except for the larger *Yersinia* heat-stable

toxin, these toxins are 18 or 19 amino acid residues in length and contain 3 intramolecular disulfide bonds. All of these toxins bind to and stimulate intestinal brush border guanylate cyclase (GTP pyrophosphate lyase, cyclizing, EC 4.6.1.2) (12-14). ST is structurally similar to guanylin, an endogenous gut peptide that binds to and activates intestinal guanylate cyclase-C receptors to regulate intestinal fluid and electrolyte transport (15). ST mimics guanylin, but with tenfold greater potency, causing life-threatening secretory diarrhea (16,17).

Protective factors in human milk. Exclusive breastfeeding up to six months after birth improves nutritional status and confers protection against diarrheal diseases (18,19). Prolonged partial breastfeeding is associated with a lower incidence of diarrhea than in children who are not breastfed; furthermore, the duration of a diarrheal episode is shorter (20) and mortality is lower in breastfed children than in those who are not breastfed (20). Promotion of breastfeeding can be one of the most cost-effective health interventions for preventing morbidity and mortality due to diarrhea (21,22).

In the past, the protection conferred by human milk was thought to be due to secretory IgA; however, in recent years, non-immune proteins, oligosaccharides, glycoconjugates (molecules such as glycolipids and glycoproteins that have an oligosaccharide moiety), and other factors such as free fatty acids and nucleotides in human milk have been recognized as protective agents.

Human milk oligosaccharides. The human milk oligosaccharide fraction, described in 1933 (23), represents approximately 12 g/L of mature milk and 22 g/L of colostrum (24) and is the third largest solid component of human milk, after fat and lactose. Over 100 major milk oligosaccharides have been isolated and their structures determined. The molecule generally has a lactose moiety at its reducing end and often contains a fucose and/or sialic acid moiety at its nonreducing end. The number of potential structural permutations for the fucosylated oligosaccharides is astronomical. Using time-of-flight mass spectrometry, Stahl (25) detected approximately 900 fucosyloligosaccharides containing up to 32 sugars and up to 15 fucose residues for the neutral oligosaccharides alone, with each having many possible isomers.

Bioactivity of human milk oligosaccharides. Oligosaccharide moieties, usually attached to glycolipids and glycoproteins, also are present on cell surfaces, where they function as receptors for factors involved in intracellular communication by binding to other cells, humoral effectors, and hormones. Pathogens bind to these same receptors, in the essential first step of infection. The glycosyltransferases that synthesize cell surface oligosaccharides are similar to those that synthesize milk oligosaccharides (26). Because of this similarity, some of the milk oligosaccharides are postulated to be structurally homologous to those on cell surfaces and might therefore bind to pathogens, inhibiting pathogen binding to glycoconjugate receptors of cells.

Infants, whose stomachs may be less acidified than those of adults and whose immune systems are not mature, may need this additional protection from enteric pathogens. We hypothesize that milk oligosaccharides are among the innate, nonimmunoglobulin factors that contribute to the protective characteristics of human milk (27,28). We have found human milk oligosaccharides or related glycoconjugates that inhibit rotavirus (29,30), *Salmonella* (31), human immunodeficiency virus (32), enterohemorrhagic *E. coli* (33), *C. jejuni* (34), and human calicivirus (35). Human milk oligosaccharides also inhibit the binding of *Streptococcus pneumoniae* to their target cells (36). Cravioto and co-workers (37) described a milk-bound oligosaccharide that inhibits adherence of enteropathogenic *E. coli* to their receptors. Other examples include the ganglioside GM_1, which binds to cholera toxin, labile toxin of *E. coli* (38), the toxin of *C. jejuni* (39), and the neutral glycolipid, globotriaosylceramide (Gb_3), which binds to different Shiga toxins (40). This proposal deals with a specific fucosylated oligosaccharide that inhibits the toxicity of ST of *E. coli* in vivo (41).

Our study of the milk glycoconjugate lactadherin and rotavirus diarrhea (30,42) showed that levels of lactadherin in milk were more strongly associated with protection than were levels of rotavirus-specific secretory antibodies. While the relative potency of the acquired and innate immune systems against any

given pathogen is of interest, the more important issue may be their redundancy and synergy resulting in comprehensive protection of the breastfed infant against a formidable array of pathogens. Within this context, the large group of relatively unexplored oligosaccharides in human milk that inhibit pathogens may represent a singular opportunity: Research in this area could lead to development of novel compounds whose protective mechanism differs radically from that of current antibiotics, to which many pathogens are already resistant.

Inhibition of ST by a human milk oligosaccharide. The seminal early work of this program project was the observation that human milk contained a non-immunoglobulin, low-molecular-weight component, absent from formula or bovine milk (43), that protected suckling mice from ST-induced diarrhea (44). This ST protective factor was isolated and characterized as follows: Pooled human milk was separated into its components; all of the ST-protective activity was localized to the oligosaccharide fraction and then to the neutral oligosaccharide fraction, specifically in the neutral oligosaccharides that bound to *Ulex europaeus*. This strongly suggested that the ST protective factor was a fucosylated oligosaccharide, because the strongest avidity of *U. europaeus* lectin is for α1,2-linked fucose structures. In tests for biological activity, all fractions were diluted to the volume of milk from which they were isolated. Under these conditions the Ulex-adherent fucosylated oligosaccharide fraction was as effective as human milk itself in preventing ST-induced diarrheal death in suckling mice (41). We next studied the mechanism of this protection, then sought to characterize the active fucosyloligosaccharide.

Mechanism of ST inhibition by the fucosyloligosaccharide. The mechanism of inhibition was studied in T84 cells, an immortal line of human enterocytes. These cells express guanylate cyclase whose extracellular domain is the receptor for ST and whose intracellular activity produces cyclic GMP, which causes loss of chloride and bicarbonate transport, ultimately leading to the efflux of fluid and electrolytes. In vivo, the result is secretory diarrhea. In the presence of the protective fucosyloligosaccharides of human milk, ST is unable to stimulate production of cyclic GMP, either in intact T84 cells or in isolated membrane preparations. The mechanism of this protection appears to be binding by the oligosaccharide to the T84 extracellular domain of guanylate cyclase, thereby blocking its binding by ST. This prevents the ST-induced loss of chloride ion homeostasis and secretory diarrhea (45).

Isolation of the ST-inhibitory fucosyloligosaccharide. The *U. europaeus*-binding fucosyloligosaccharide fraction was resolved into more than 30 components by semi-preparative HPLC. Of these fractions, only one displayed consistent, robust activity at the concentration found in human milk. This fraction was further subfractionated into seven components of which only one (now identified as a trifucosyl-*iso*-lacto-*N*-octaose – TF*i*LNO) inhibited the diarrheagenic activity of stable toxin in the suckling mouse. We estimate that this subfraction is active at a concentration of approximately 30 ppb, the concentration at which it is found in human milk (46). The total oligosaccharides are approximately 1% of human milk, thus the active TF*i*LNO represents approximately 0.000003% of the oligosaccharides in milk.

Heterogeneity of oligosaccharides in human milk. Milks from different women do not contain identical oligosaccharides, and they vary in their protective capacities against disease. During our laboratory studies of ST inhibition by oligosaccharides we observed that milk from different individuals varied greatly in inhibitory capacity. These differences are genetically based.

Fucosyltransferase products of the secretor and Lewis gene family synthesize 2-linked fucosyloligosaccharides. An early study by Viverge classified women of European origin into three groups on the basis of the fucosyloligosaccharides of their milk (47,48). Milks from the first group (Le^{a-b+}, secretors – approximately 75% of the population) contained the highest concentration of fucosyloligosaccharides with a preponderance of α1,2-linked fucose moieties. A second group of mothers (blood type Le^{a+b-}, nonsecretors – about 20% of the population) produced milk whose fucosyloligosaccharides contain α1,3 and α1,4-linked fucose, but not α1,2-linked fucose. The remaining 5% of the population produced milk that contained the lowest amounts of fucosyloligosaccharides, with structures quite different from those of the other two groups. Studies in other populations have associated Lewis and secretor gene expression with milk oligosaccharide

composition (49). Populations of non-European origin can have different expression of the Lewis blood group types and different expression of oligosaccharides in their milk (50,51).

C. Progress Report

As of the beginning of the last funding cycle, the ability of human milk oligosaccharides to protect against stable toxin (ST) of enterotoxigenic *E. coli* (ETEC) had been established, in vitro and in vivo, and the protective factor had been isolated to a subfraction of 2-linked fucosyloligosaccharides, TF*i*LNO. During the most recent funding cycle our efforts focused on the clinical relevance of this protective fucosyloligosaccharide and on the structure of the active component.

Human milk protects breastfed infants from ST-associated diarrhea. Our epidemiologic data are from a population of about 586 mother-infant dyads, living in San Pedro Martir, Mexico City, and prospectively studied from 1988–1991 (See Epidemiology Core). We found an inverse relationship between breastfeeding and risk of ST-ETEC diarrhea. The relative risk of ST-ETEC diarrhea among children who were not breastfeeding was 0.27. For those who were partially breastfed the relative risk was 0.18, and for those whose source of nutrients was exclusively human milk the risk was 0.09. This protection was strongest during the first three months of life, intermediate between months 4 and 6, and least between months 7 through 12. These data are consistent with an oligosaccharide of human milk inhibiting ST-associated diarrhea and with levels of the oligosaccharide declining over the course of lactation. Because secretory IgA also decreases over the course of lactation, we designed experiments to more fully define the variation in human milk oligosaccharide expression and its relationship to risk of diarrhea in breastfeeding infants.

Variations in human milk composition. We had observed in the laboratory that human milk samples varied in their capacity for inhibiting ST and contained different amounts of the oligosaccharides of interest. In order to study relationships between human milk and disease, we needed to understand these variations and their biological implications.

First we defined the variation in expression of milk oligosaccharides among a population of 50 lactating mothers in the typical Mexican population (52). Figure 1 shows that the expression of 2-fucosyltransferase activity in mature human milk (65±13 days of lactation) was quite heterogeneous, resulting in widely differing concentrations of fucosylated oligosaccharides as is evident by the large standard deviations in Table 1.

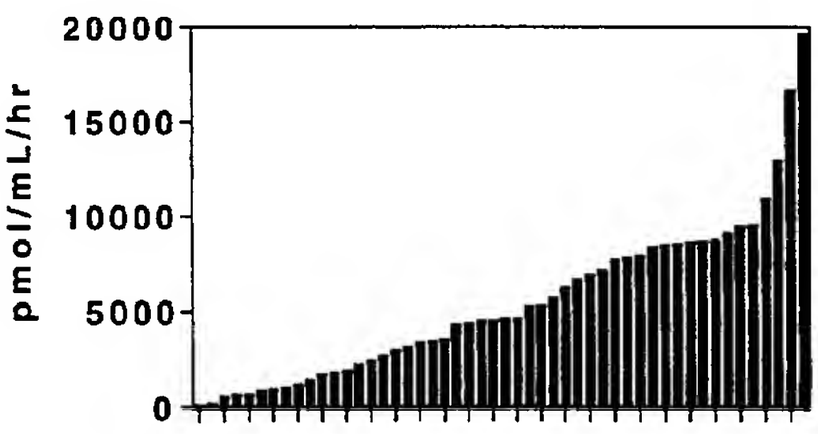


Figure 1. *2-Fucosyltransferase activity in mature milk of 50 individual mothers*

Table 1. Concentrations of oligosaccharides in milk from 50 donors

Oligosaccharide	Mean±SD (mg/L)
LNT	400±350
LNneoT	180±140
2'-FL	1,210±560
3-FL	360±280
LDFT	300±420
LNF-I	940±850
LNF-II+III	640±560
LDFH-I	550±780

We next asked whether the expression of fucosyloligosaccharides in milk produced by a given individual changes over the course of lactation. We obtained milk samples from 12 mothers at intervals over the first 12 months of lactation. Eleven of the mothers were secretors; because the secretor gene expresses a major 2-fucosyltransferase, their milk contained 2-linked fucosyloligosaccharides. Figure 2 (next page) demonstrates that the total amount of oligosaccharides in the milk of these 11 mothers decreased over the course of lactation, primarily due to a decrease in the production of 2-linked fucosyloligosaccharides. The ratio of all

oligosaccharides containing 2-linked fucose to oligosaccharides containing only 3- and 4-linked fucose was calculated (Figure 3). Surprisingly, this ratio decreased exponentially toward unity over the course of lactation, indicating some form of coordinated reciprocal regulation of the enzyme activities responsible for the synthesis of the different types of fucosyloligosaccharides.

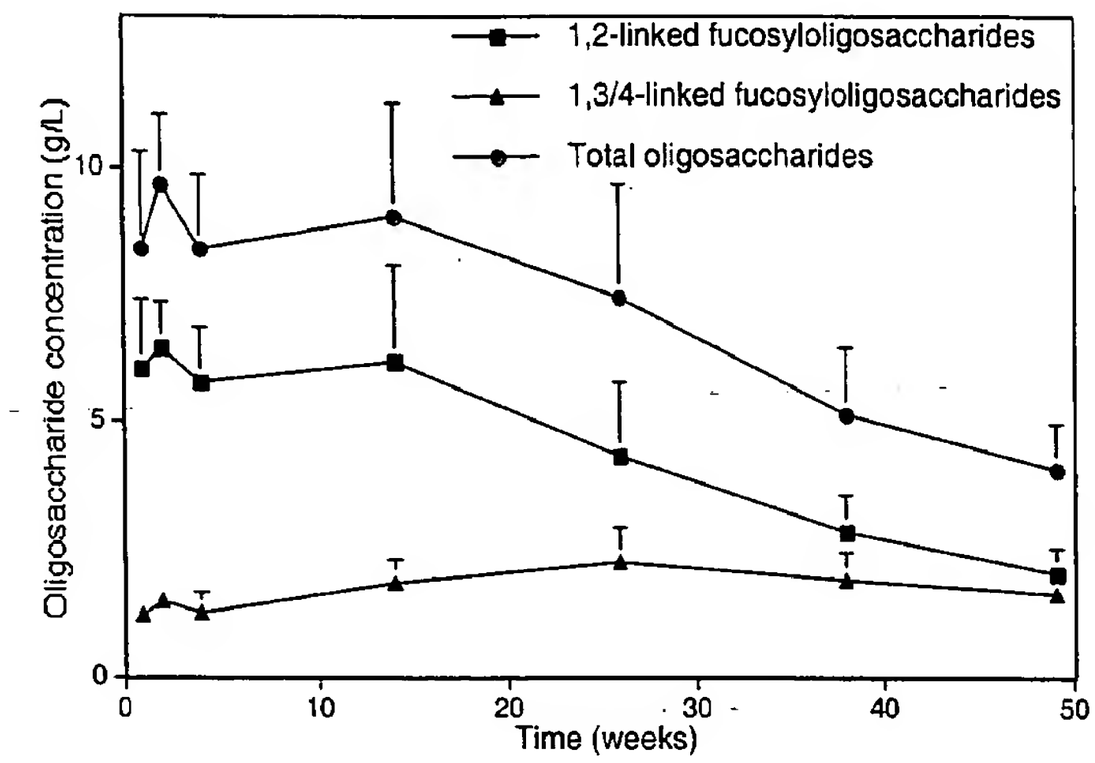


Figure 2. *Fucosyloligosaccharide levels over lactation*

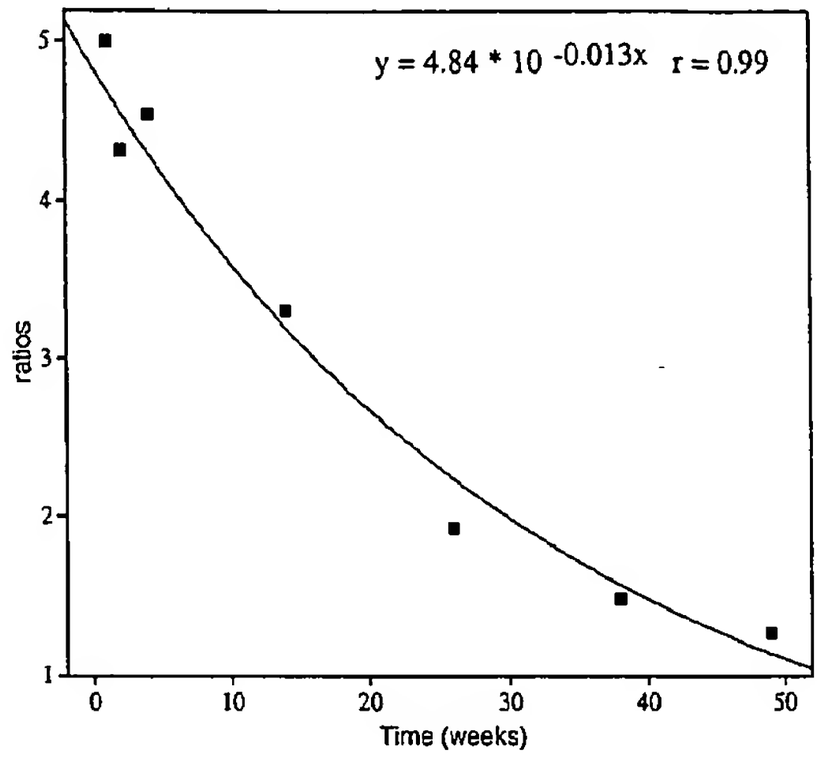


Figure 3. *Oligosaccharide ratios over lactation*

The average concentrations of the individual fucosyloligosaccharides in milk from these 11 mothers are shown in Figure 4. The dominant oligosaccharides were 2'-FL and LNF-I (see Table 2 for structures and abbreviations), both direct products of 2-fucosyltransferase activity.

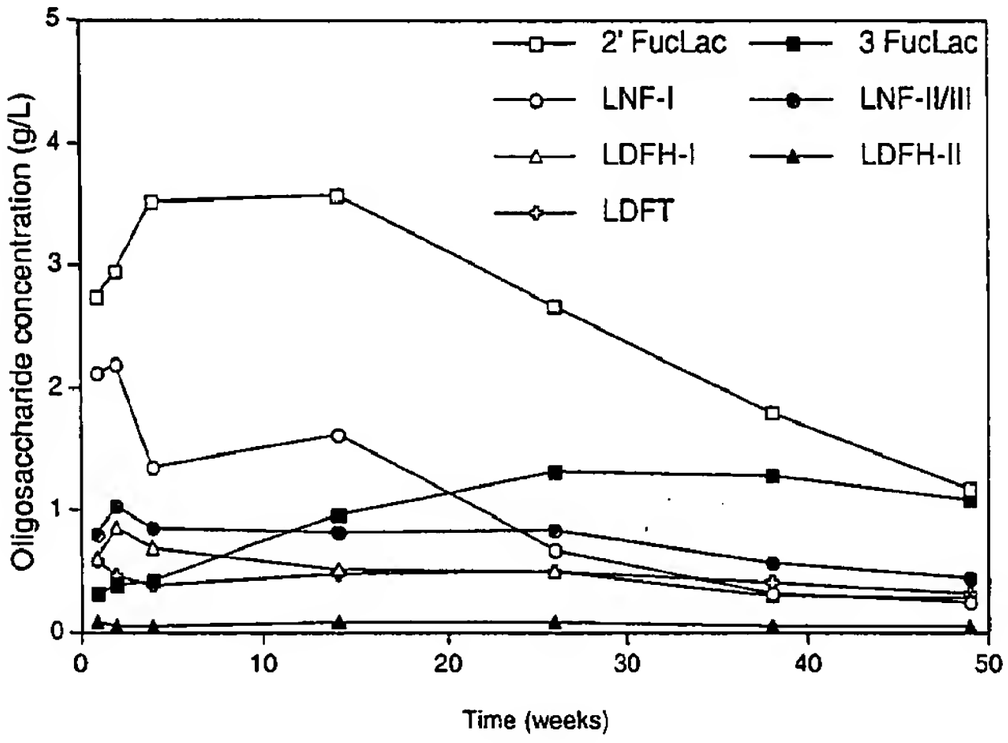


Figure 4. *Individual oligosaccharides: secretor*

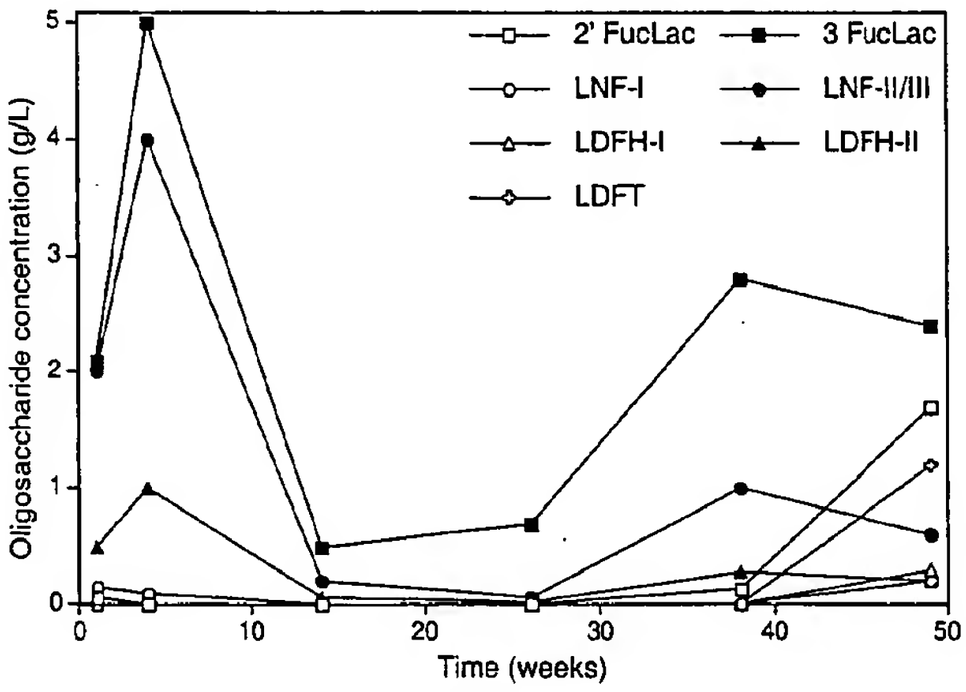


Figure 5. *Individual oligosaccharides: non-secretor*

Figure 5 shows the concentrations of oligosaccharides in the 12th mother, who was a nonsecretor (homozygous recessive for the secretor gene). 2-Linked fucosyloligosaccharides were almost completely absent from her milk through the first 30 weeks (7 months) of lactation, but the amounts of 3-, and 4-linked fucosyloligosaccharides were relatively higher, especially early in lactation. Surprisingly, 2-linked fucosyloligosaccharides appeared in the milk of this non-secretor after 30 weeks of lactation. This heretofore unrecognized increase in 2-linked oligosaccharides late in lactation could be due to increased activity of the *FUT1* gene product, which is also a 2-fucosyltransferase. The ratio of 2-linked fucosyloligosaccharides to those containing only 3- and 4-linked fucose, which was originally close to zero for this nonsecretor, approached unity after 30 weeks. The implication is that, in both secretors and nonsecretors, the early and

late production of 2-linked fucosyloligosaccharides arise from two different mechanisms. These hypotheses can be tested through molecular biological techniques as proposed in specific aim 4 of this project.

The finding that the oligosaccharide content of human milk varies dramatically over the course of lactation contradicted the long-standing supposition that oligosaccharide concentrations remain relatively constant, and had important ramifications for the physiology of lactation. Comparison of milk from different women at the same stage of lactation revealed that if the women were ranked according to the relative proportions of specific oligosaccharides in their milk, that rank order remained fairly constant despite the changes in milk oligosaccharide content over time. This finding suggested that the relative ranking of each mother was based on intrinsic genetic traits. Because the oligosaccharide content of milk changes over the course of lactation, milk samples can be obtained during a fixed stage of lactation, ranked, and then the rank of each mother can be related to diarrhea episodes in her infant over the entire period of lactation.

Associations between milk oligosaccharides and infant diarrhea. We designed a series of experiments in which we measured the milk oligosaccharides during a standardized early period of lactation. Milk samples were obtained during weeks 2 - 5 postpartum from 93 mothers, and the concentrations of individual oligosaccharides were measured in each sample. Table 2 shows the results expressed as μmoles per liter of milk and as percentage of the total measured oligosaccharides. We compared the standard errors

Table 2. Major human milk oligosaccharides (OS) in the first 5 weeks of lactation

Type	Name	Structure	*μmol/L*	*% OS*
1	LNT, Lacto-*N*-tetraose	Galβ(1→3) GlcNAcβ(1→3) Galβ(1→4) Glc	1273±82	10.1±0.5
1	LNF-I, Lacto-*N*-fucopentaose-I	Fucα(1→2) Galβ(1→3) GlcNAcβ(1→3) Galβ(1→4) Glc	3213±181	25.0±1.1
1	LNF-II, Lacto-*N*-fucopentaose-II	Galβ(1→3)↘ GlcNAcβ(1→3) Galβ(1→4) Glc Fucα(1→4)↗	1149±83	8.9±0.4
2	LNF-III, Lacto-*N*-fucopentaose-III	Galβ(1→4)↘ GlcNA β(1→3) Galβ(1→4) Glc Fucα(1→3)↗		
1	LDFH-I, Lacto-*N*-difucohexaose-I	Fucα(1→2) Galβ(1→3)↘ GlcNAcβ(1→3) Galβ(1→4) Glc Fucα(1→4)↗	1256±104	9.5±0.6
2	LNneoT, Lacto-*N*-neotetraose	Galβ(1→4) GlcNAcβ(1→3) Galβ(1→4) Glc	415±27	3.3±0.2
2	2'-FL, 2'-Fucosyllactose	Fucα(1→2) Galβ(1→4) Glc	3854±108	33.7±1.1
2	3-FL, 3-Fucosyllactose	Galβ(1→4)↘ Glc Fucα(1→3)↗	577±100	4.3±0.5
2	LDFT, Lactodifucotetraose	Fucα(1→2) Galβ(1→4)↘ Glc Fucα(1→3)↗	698±75	5.2±0.4

Mean±SE; *n*=93; LNF-II and LNF-III coelute and are measured together as a single peak. Percent OS equals the concentration of each oligosaccharide divided by the concentrations of all of the oligosaccharides measured x 100. The oligosaccharides measured account for over 90% of the weight of the total oligosaccharides in human milk.

associated with each of these measures and found that the average variation for the absolute concentration of the oligosaccharides was 8% of the mean, while the average variation for an oligosaccharide as the percentage of all oligosaccharides was 6%. This 25% reduction in the coefficient of variation by using total oligosaccharides as a denominator may be due to its ability to correct for extraneous variability due to factors like desiccation in the freezer, recovery during sample preparation, etc. This more precise measure of

individual oligosaccharides as a percentage of total oligosaccharides was used to investigate the relationship between the relative amounts of these oligosaccharides in milk and the protection afforded the breastfeeding infants against diarrhea and specifically against campylobacter-associated and calicivirus-associated diarrheas. Milk containing high levels of specific oligosaccharides was being consumed by infants who had fewer cases of moderate-to-severe diarrhea (see Epidemiology Core). The oligosaccharides most strongly associated with protection were LNF-I (H-I antigen) ($P<0.001$), 2'-FL (H-2 antigen) ($P=0.001$) and LDFH-I (Le^b antigen) ($P=0.002$). When the cases of diarrhea were segregated according to pathogen, high levels of 2'-FL (H-2 antigen) were significantly associated with less campylobacter diarrhea ($P=0.004$), while high levels of LDFH-I (Le^b) were associated with a lower incidence of diarrhea due to calicivirus ($P=0.012$). These data strongly support the clinical relevance of our earlier laboratory findings of specific human milk oligosaccharides inhibiting specific enteric pathogens in vitro, in vivo, and ex vivo.

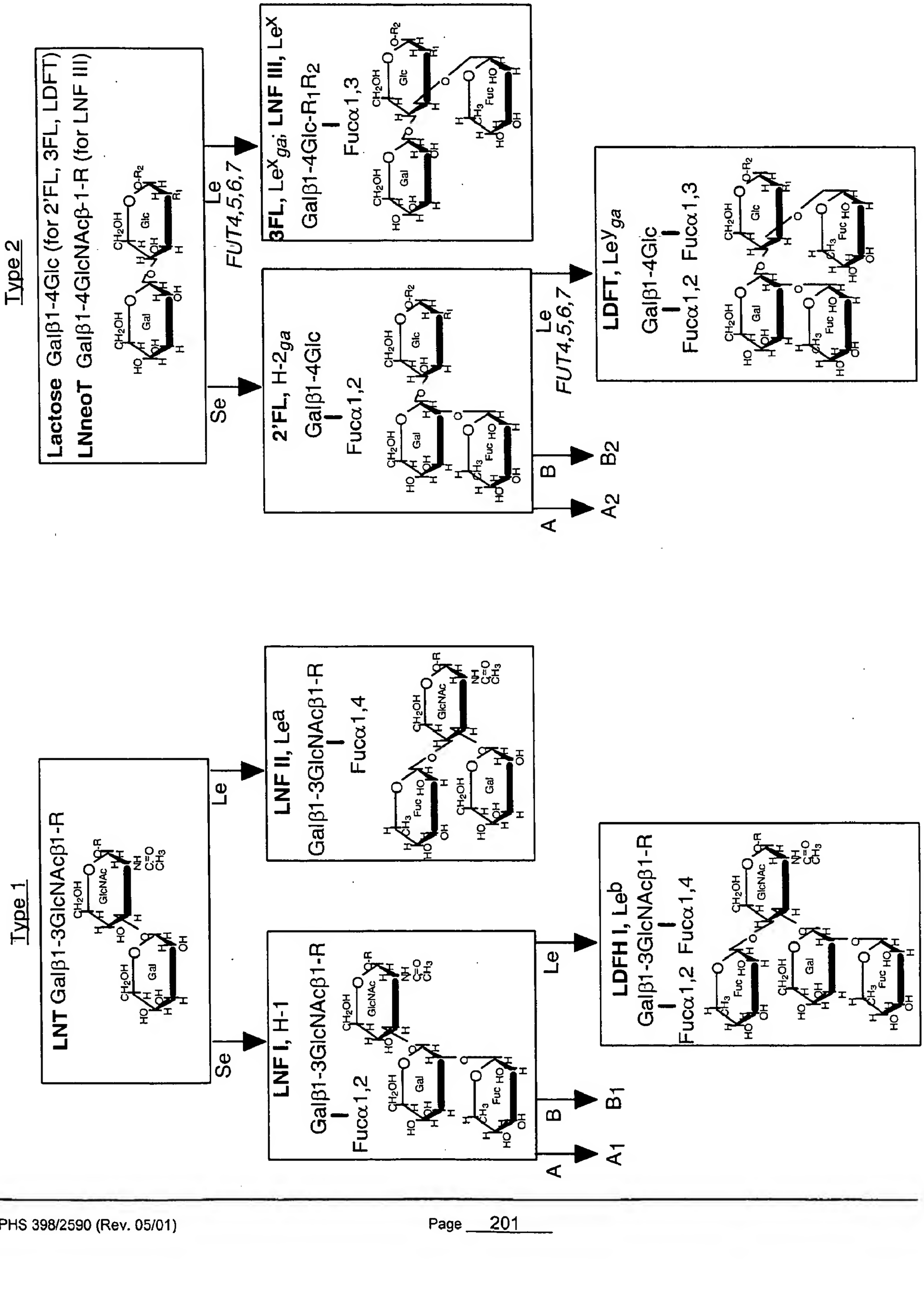
Relationship of Lewis and secretor genes to variation of milk oligosaccharides. Our isolation of a milk component (trifucosyl-*iso*-lacto-*N*-octaose) that inhibits ST activity indicated that these nine major simple oligosaccharides could be biomarkers for the presence of other, less abundant protective oligosaccharides whose syntheses are controlled by the same fucosyltransferases as those oligosaccharides that are associated with protection.

Two extensively studied mammary fucosyltransferases thought to control the synthesis of human milk fucosyloligosaccharides are a 2-fucosyltransferase (FucT-II, the product of the secretor gene *FUT2*) and 3/4-fucosyltransferase (FucT-III, the product of the Lewis gene *FUT3*). Other investigators have proposed that FucT-III acts primarily as a fucosyl α1,4-transferase and only secondarily, if at all, as a fucosyl α1,3-transferase in the synthesis of milk oligosaccharides. The fucosyl α1,3-transferase function is thought to be met by α1,3-fucosyltransferase products of the Lewis gene family, perhaps one or more FucT-IV, FucT- V, FucT- VI or FucT- VII (53).

The interrelationship of these gene products in the synthesis of the major milk oligosaccharides is shown in Figure 6 (next page). The major human milk oligosaccharides, measured in our epidemiologic studies, are arranged according to their core structures (type 1 and type 2) and their place in the synthetic scheme for secretor and Lewis structures. The core type 1 structure, lacto-*N*-tetraose (LNT), is Galβ1,3GlcNAc on the terminal end of lactose (-R). The core for the most abundant type 2 structures in milk includes lactose (for 2'-FL, 3-FL, and LDFT), lacto-*N*-neo-tetraose (for LNneoT), and Galβ1,4GlcNAc on a lactose terminus (for LNF-III). Lewis structural moieties are based on a backbone ending in Gal-GlcNAc; however, the most prevalent type 2 structures in human milk contain lactose (Gal-Glc) and therefore are defined as the glucose analogs (*ga*) to the type 2 Lewis structures, where $-R_1$ is –OH and $-R_2$ is –H. True Lewis structures, such as LNF-III, have an R1 of *N*-acetyl and an R2 of lactose or lactosamine. Abbreviations for the fucosyltransferase genes are: Se (secretor gene, *FUT2*), Le (Lewis gene, *FUT3*), and *FUT4*,5,6,7 (Lewis gene family of 3-fucosyltransferases). Blood group A and B structures, synthesized from H-1 and H-2 antigens, are not major components of milk oligosaccharides, and only a few of these A or B moieties have been described in milk oligosaccharides.

Because the expression of Lewis and secretor genes is also the basis for the Lewis blood group types, the determination of the Lewis blood group phenotypes of lactating mothers allowed us to infer the relationship between milk oligosaccharides and Lewis and secretor genes. Using hemagglutination of erythrocytes, we determined Lewis blood group types of a population of mixed indigenous and European ancestry in San Pedro Martir, Mexico City. This population has a much lower prevalence of nonsecretors than would a population of European origin. All of the 93 donors had some 2-linked fucosyloligosaccharides in their milk

Figure 6. The major fucosylated oligosaccharides of human milk arranged according to their Lewis structure



and had the Lewis blood group types of obligate secretors. The frequency of their Lewis blood group phenotype fit the following Hardy-Weinberg distribution (Table 3A).

Table 3A. Hardy Weinberg distribution of Se and Le genotype

Secretor (*FUT2*) genotype (frequency: se=.15, Se=.85)

Lewis (*FUT3*) genotype (frequency: le=.5, Le=.5)	sese	Sese	SeSe	Total
lele	a-b- (.005)	a-b- (.065)	a-b- (.18)	0.25
Lele	a+b- (.01)	a-b+ (.13)	a-b+ (.36)	0.50
LeLe	a+b- (.005)	a-b+ (.065)	a-b+ (.18)	0.25
Total	0.02	0.26	0.72	

Table 3B. Predicted and observed frequencies of Lewis phenotype

	Hardy-Weinberg Prediction	Observed in study population
a+b-	2%	2%
a-b+	73%	72%
a-b-	25%	26%

n = 93

The gene frequency of .85 for Se, the dominant secretor gene, fits well with the observation that most of these mothers are secretors. There are also "partial secretors," as defined in the Core, who have an Se mutation that allows very limited expression of FucTII activity in homozygous recessive individuals. .The frequency of .5 for Le, the dominant Lewis gene, fits well with our finding a 3 to 1 ratio of Le^{a-b+} to Le^{a-b-}. Table 3B predicts a distribution of Lewis phenotypes in this population that almost exactly matches our observed values. The phenotype Le^{a-b-} is a manifestation of the genotype lele (i.e., homozygous recessive for the Lewis gene). These individuals express less 3- and 4-linked fucose on their red cell glycoconjugates than do women of the Le^{a-b+} phenotype. The patterns of expression of human milk oligosaccharides were arranged by Lewis blood group phenotype of the mother in an attempt to understand the genetic basis of the variation of milk oligosaccharides. As can be seen in Table 4, these individuals, who expressed less 3- and 4-linked fucose on their red cells, also expressed less of the 3- and 4-linked fucose-containing oligosaccharides (Le^a, Le^b, Le^x, Le^y) in their milk. Conversely, the phenotype Le^{a-b+} is a manifestation of the genotypes that contain one or two dominant Lewis genes and one or two dominant secretor genes. Because the Lewis fucosyltransferase consumes 2-fucosylated structures to produce the combined 2-, 3-, and 4-linked fucosylated structures, the phenotype is negative for Lewis a but positive for Lewis b antigens in red blood cells. On the surfaces of their erythrocytes, these individuals express more fucosyloligosaccharide moieties containing exclusively 3- and 4-linked fucose, and likewise, their milk contains a higher proportion of 3- and 4-linked fucosyloligosaccharides than is found in milk from women with the Le^{a-b-} phenotype.

Consistent with the Lewis synthetic pathway shown in Figure 6, Table 4 demonstrates a reciprocal relationship between the 2-linked fucosyloligosaccharides and fucosyloligosaccharides that contain 3- and 4-linkages as they compete for common substrates. The ratio of the 2-linked oligosaccharides ($H+Le^b+Le^y$) to those containing only 3- and 4- linkages (Le^a+Le^x) defines this relationship for human milk in a single measure. Table 4 illustrates that this ratio is significantly higher in milk produced by Le^{a-b-} mothers than in milk produced by Le^{a-b+} mothers.

Table 4. Milk oligosaccharides by maternal Lewis blood group type

% milk oligosaccharides (Mean±SE)

Oligosaccharide (Lewis antigen)	Maternal Lewis Blood Group Le^{a-b+} *(n=67)*	Le^{a-b-} *(n=24)*	p
LNF-I (H-1)	23±1	30±2	.003
2'-FL (H-2)	31±1	42±2	<.001
Total H	**54±1**	**72±2**	**<.001**
LDFH-I (Le^b)	11±1	4±1	<.001
LDFT (Le^y)	6±0	4±1	.028
Total Le^b+Le^y	**17±1**	**8±1**	**<.001**
LNF-II,-III (Le^a,Le^x)	10±0	5±0	<.001
3-FL (Le^x) type 1	5±1	2±0	<.001
Total Le^a+Le^x	**15±1**	**7±1**	**<.001**
Ratio	5.3±0.4	13.3±2.1	<.001

Mean±SE. p of the difference between Le^{a-b+} and Le^{a-b-} by ANOVA and Student's *t* test; *n*=91

Ratio=(H+Le^b+Le^y) / (Le^a+Le^x)

Table 5. Oligosacchairde ratios by blood group type

Quartile of milk oligosaccharide ratio	Lewis blood group type a– b+	a– b–
Lowest, *n* (%)	23 (100)	0 (0)
Middle, *n* (%)	38 (81)	7 (15)
Highest, *n* (%)	6 (26)	17 (74)

*Fisher's exact test, p<0.01

These ratios were sorted from the highest to the lowest and grouped by quartile. The distribution of these ratios according to Lewis blood group phenotype of the mother is asymmetric, as shown in Table 5. All 23 mothers whose milk was in the lowest quartile belonged to Lewis blood group type Le^{a-b+}; conversely, 74% of the mothers whose milk oligosaccharide ratios were in the highest quartile belonged to Lewis blood group type Le^{a-b-}. The 23 mothers whose milks were in the lowest quartile represent approximately one third of the mothers of the Le^{a-b+} blood group phenotype. The genotypes that would be expected to have the lowest ratios of 2-linked to other fucosyloligosaccharides are SeseLeLe, (double box in Table 3a) whose ratio of secretor to Lewis enzyme is low, and to a lesser extent SeSeLeLe or SeseLele, also bolded in Table 3a, whose gene dose is equal, but could favor Le activity because Le has a higher affinity for substrate than Se, favoring formation of its products.

Relationship of milk fucosyloligosaccharide ratios to ST diarrhea in nursing infants. If the enzymes (i.e., secretor and Lewis gene products) that control the synthesis of the 2-linked fucosyloligosaccharides early in lactation likewise control the synthesis of the particular 2-linked fucosyloligosaccharide structure that protects against ST, then one would predict a relationship between the ratios of 2- to 3/4-linked oligosaccharides and the incidence of ST-associated diarrhea in breastfeeding infants. A high ratio would indicate a relatively higher activity of 2-fucosyltransferases and more of the protective oligosaccharide in the milk. To test this hypothesis, we ascertained this ratio in milks of mothers whose breastfed infants were grouped according to their status with regard to ST infection: Group 1, infants in whom infection by ST-producing *E. coli* was not detected; Group 2, infants asymptomatically infected with ST-producing *E. coli*; Group 3, infants infected with ST-producing *E. coli* and suffering diarrhea. Milk consumed by asymptomatic

infants had the same oligosaccharide ratios as did the milk consumed by uninfected control infants. Milk consumed by infected infants with diarrhea had significantly lower ratios of 2- to 3- and 4-linked fucosyloligosaccharide (Figure 7).

Thus, even in this population of mothers who are secretors and all produce 2-linked fucosyloligosaccharides, lower relative amounts of these 2-linked oligosaccharides were associated with increased risk of ST-associated diarrhea. One might expect that the protection against ST afforded by human milk might show even greater variation in a population that includes nonsecretors (populations of European origin typically contain 20% nonsecretors). These data, taken together with the finding that high levels of 2-fucosyllactose in milk are associated with decreased risk of campylobacter-associated diarrhea and high concentrations of LDFH-I in milk are associated with decreased risk of calicivirus-associated diarrhea in breastfed infants, imply that human milk oligosaccharides are an important part of an innate immune system of human milk that protects breastfed infants from enteric and other diseases. More specifically, these data support the clinical relevance of a 2-linked fucosylated oligosaccharide as a significant agent against ST-associated diarrhea.

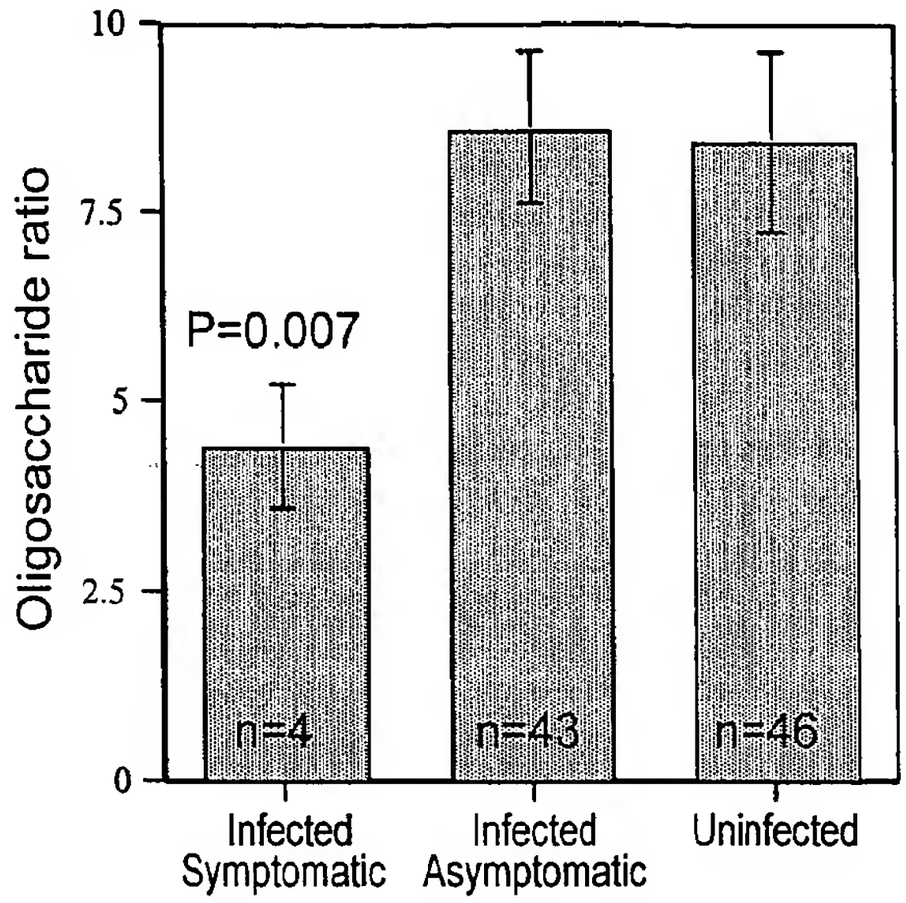


***Figure 7**. Milk oligosaccharide ratios and ST diarrhea in breastfed infants*

Isolation and bioactivity assay of ST-inhibitory fucosyloligosaccharide. These encouraging clinical results persuaded us to determine the minimum structural features of these oligosaccharides that are required for this protection.

The structure of the ST-inhibitory fucosyloligosaccharide was evaluated as follows: Compositional data from GC/MS of permethylated alditol acetates, elution times in normal and reversed-phase HPLC, TLC, chromatography on BioGel P-4, FAB MS, and MALDI-TOF MS all indicated a sugar composition of 11 or 12 sugar residues, with a composition of $Fuc_{3/4}Gal_4GlcNAc_3Glc$. Electrospray MS/MS indicated that this highly purified inhibitory fraction contained trifucosylated and tetrafucosylated *iso*-lacto-*N*-octaose structures.

Hoping to identify molecules that might contain sufficient structural homology with the ST-inhibitory oligosaccharide to exhibit ST-inhibitory activity, we isolated a series of novel fucosyloligosaccharide fractions and pure fucosyloligosaccharides from human milk; each contained within its structure a moiety of at least 10 sugars with at least 2 of them being fucose. The biological activity of these fractions (Table 6, next page) was tested in the suckling mouse model. ST was administered by orogastric intubation to three-day old C57BL/6J mice along with an oligosaccharide in a total volume of 0.03 mL (30 μL) of isotonic PBS. The ability of the oligosaccharide to prevent diarrhea-induced death by ST after eight hours is the dependent variable. Because the novel fucosyloligosaccharide(s) would probably be less active than the actual inhibitor, the molecules were tested at 400 mg/L, five orders of magnitude greater than the estimated concentration (30-40 ppb) of the native ST-inhibitory fucosyloligosaccharide in milk. Human milk oligosaccharides, tested at their concentration in milk, served as positive controls.

The data in Table 6 show that compounds whose structures have homology to trifucosyl-*iso*-lacto-*N*-octaose (TF*i*LNO) with terminal fucosyl α1,2 linkages seem to be most able to inhibit the ability of stable toxin to cause diarrhea at these concentrations.

Table 6. Inhibition of ST in suckling mice by human milk oligosaccharides

	Dead	Alive	P
Stable toxin	39	6	
Difucosyl-*iso*-lacto-*N*-decaose (1)	11	0	ns
Difucosyl-*iso*-lacto-*N*-decaose (2)	11	0	ns
Difucosyl-*iso*-lacto-*N*-decaose (3)	13	0	ns
Difucosyl-*iso*-lacto-*N*-decaose (4)	12	0	ns
Difucosyl-*iso*-lacto-*N*-decaose (5)	12	0	ns
Tetrafucosyl-*para*-lacto-*N*-octaose Galβ1-3GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Glcβ1-4Gal Fucα1-2↗Fucα1-4↗ Fucα1-3↗ Fucα1-3↗	8	1	ns
Trifucosyl-*iso*-lacto-*N*-octaose(2) Fucα1-3↘ Galβ1-3GlcNAcβ1-3Galβ1-4GlcNAc β1-6↘ Galβ1-4Glc Galβ1-3GlcNAcβ1-3↗ Fucα1-2↗Fucα1-4↗	9	0	ns
Tetrafucosyl-*iso*-lacto-*N*-octaose(2) Fucα1-4↘ Fucα1-3↘ Galβ1-3GlcNAcβ1-3Galβ1-4GlcNAcβ1-6↘ Galβ1-4Glc Galβ1-3GlcNAcβ1-3↗ Fucα1-2↗Fucα1-4↗	15	8	0.03
Pentafucosyl-*iso*-lacto-*N*-octaose Fucα1-2↘Fucα1-4↘ Fucα1-3↘ Galβ1-3GlcNAcβ1-3Galβ1-4GlcNAcβ1-6↘ Galβ1-4Glc Gal β1-3GlcNAcβ1-3↗ Fucα1-2↗Fucα1-4↗	8	6	0.02
Tetrafucosyl-*iso*-lacto-*N*-octaose(1) Fucα1-2↘ Fucα1-3↘ Galβ1-3GlcNAcβ1-3Galβ1-4GlcNAcβ1-6↘ Galβ1-4Glc Gal β1-3GlcNAcβ1-3↗ Fucα1-2↗Fucα1-4↗	6	6	0.01
Difucosyl-*iso*-lacto-*N*-octaose(3) Fucα1–2↘ Galβ1-3GlcNAcβ1-3Galβ1-4GlcNAcβ1-6↘ Galβ1-4Glc Galβ1-3GlcNAcβ1-3↗ Fucα1–2↗	4	6	0.004
Total oligosaccharides	7	6	0.02

p is for the comparison to stable toxin alone by Fisher's exact test
Fractions 1–5 are mixtures of large fucosylated oligosaccharides

Summary. The results obtained over the last funding period have led to several important new conclusions:

- The amounts and types of oligosaccharides vary widely among human milks, consistent with the known heterogeneity of human glycoconjugate expression.
- The heterogeneity of fucosyloligosaccharide expression in milk is strongly related to individual variation in the Lewis blood group type, reflecting the genetic polymorphism of the Lewis and secretor genes.
- The milk oligosaccharides of individual mothers vary quantitatively and qualitatively across the first year of lactation to an extent heretofore unsuspected.
- Milk fucosyltransferase activities appear to undergo coordinated, reciprocal changes during the course of lactation.
- High levels of specific fucosyloligosaccharides in milk are strongly associated with protection against diarrhea caused by specific pathogens.
- A larger amount of 2-linked relative to 3- and 4-linked fucosyloligosaccharides is associated with protection from ST-*E. coli* diarrhea. The oligosaccharide from milk that protects against ST contains 2-linked fucoses on a biantennary octaose core.

In conclusion, the human milk oligosaccharides represent a major component of the innate immune system of human milk, which seems to be a major defense of nursing infants against disease, and especially against pathogens to which the infant is naïve. Such protection against ST-producing *E. coli* in nursing infants is lower in the milk of mothers of particular genotypes that express lower relative amounts of 2-linked fucose.

Publications. See Core section.

D. Research Design and Methods

These studies are designed to define structural determinants of fucosyloligosaccharides that have ST-inhibitory activity to compare the relative efficacy of various structural determinants, and to define the mechanism of ST inhibition. Simultaneous studies will investigate the genetic basis for the expression of the ST inhibitor, and thus elucidate differences in the protective capacities of milk from different women.

We will synthesize a complete library of structural motifs that could be present in human milk fucosyloligosaccharides, with priority on testing those that occur in the protective trifucosylated octaose. As these structural motifs are synthesized, they will be tested for ST-inhibitory activity, individually and in combination, in the suckling mouse model. We already know most of structural motifs of the naturally occurring, highly potent component of human milk that inhibits binding of ST to human enterocytes in vitro and inhibits ST-induced diarrhea in vivo.

The activity of the most active oligosaccharide or mixture of oligosaccharides will be compared directly with that of the fucosylated milk oligosaccharide fraction, which contains the naturally occurring ST inhibitor. The mechanism whereby these novel structures inhibit the activity of ST will be studied in human enterocytes (T84 cells) and compared with that of the naturally occurring trifucosylated octaose fraction from human milk.

Because ST binds to the same guanylate cyclase C receptor as does guanylin, an endogenous intraluminal hormone that helps regulate fluid and electrolyte balance (54), we will test the novel hypothesis that the fucosyloligosaccharide that inhibits ST could also play a physiological role by modulating this guanylin receptor. This information is crucial if milk oligosaccharides are to be developed as a possible therapy for diarrhea. Using T84 cells, we will determine if milk oligosaccharides inhibit guanylin-induced activation of guanylate cyclase and guanylin binding to cell membranes.

In concert with the Glycobiology, Epidemiology, and Molecular Biology cores, the genetic basis of heterogeneous expression of ST-inhibitory human milk oligosaccharides will be determined in our prospectively studied cohort in San Pedro Martir. The results of genetic studies can potentially provide ways to identify children who are at increased risk for ST-associated diarrhea. The successful completion of this

project would provide additional support for breastfeeding promotion, and possibly development of supplements to protect infants at risk.

Specific Aim 1. Synthesize a complete combinatorial library of fucosylated trisaccharides and tetrasaccharides that encompass the possible ultimate products of the human milk fucosyltransferases of the Lewis and secretor gene family

We will use two approaches for the synthesis of a combinatorial library of unbranched fucosyloligosaccharides that encompass the possible products of the Lewis (and secretor) gene family. The first utilizes a scheme of automated synthesis devised by Dr. Peter Seeberger of Department of Chemistry at the Massachusetts Institute of Technology, Cambridge, MA. His laboratory is widely recognized as being among the most advanced in the synthesis of carbohydrate structures and is able to automate the synthesis of many oligosaccharides (55). The second approach, employing conventional batch synthesis using well-established techniques, will be used when automated synthesis is not feasible. Dr. Seeberger has expressed strong enthusiasm for consulting for our project (see attached letter) and will consult with Glycobiology Core personnel engaged in these syntheses.

Automated Synthesis

The methods developed for the automated assembly of complex oligosaccharides will be used to produce oligosaccharide structures believed to be responsible for protection from the stable toxin of *E. coli*. For the proposed work, the initial syntheses will be of those oligosaccharide structures most similar to the natural protective agent, followed by the syntheses of the remainder of the combinatorial library to determine the most potent combination of structures.

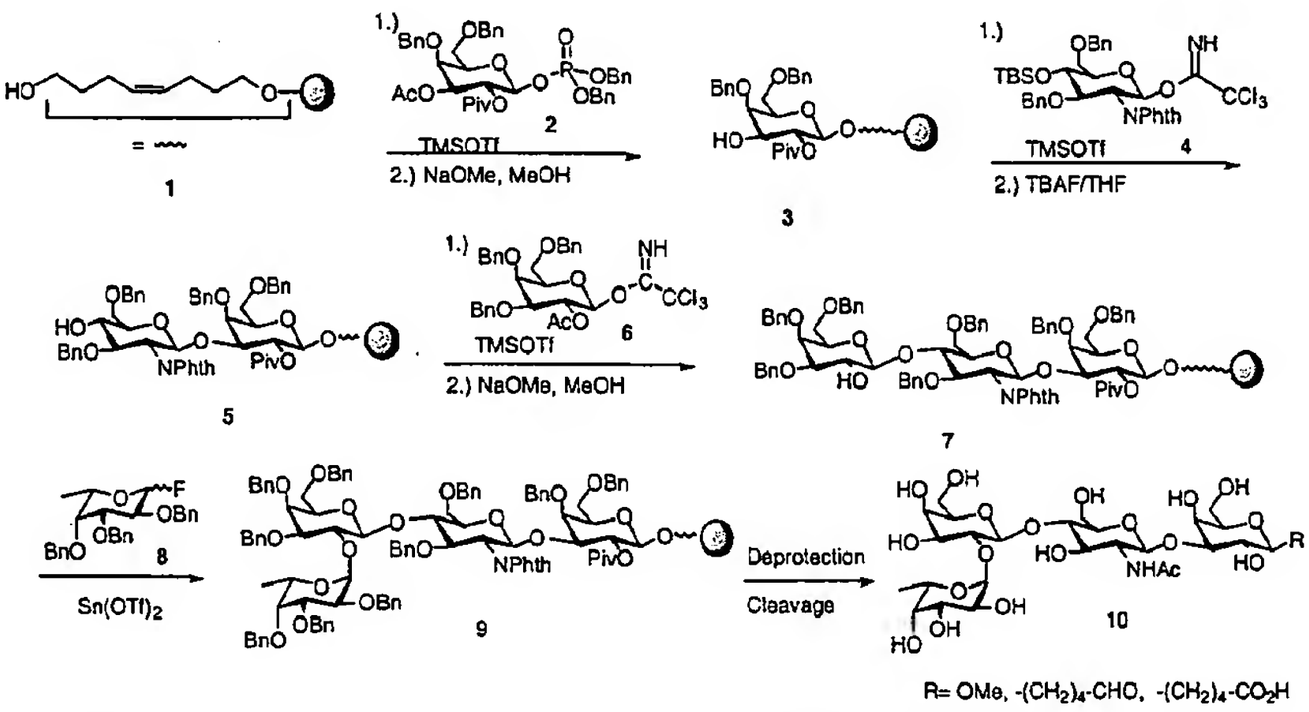


Figure 8. *Solid-phase synthesis of the H-2 tetrasaccharide*

Approach 1: Automated synthesis of the H-1 and H-2 tetrasaccharides. Initially, the synthetic effort will focus on the preparation of the H-1 and H-2 tetrasaccharides on a solid support as outlined for H-2 in Figure 8. The intermediate for H-2 is a penultimate Galβ1,4GlcNAc. The synthesis for H-1 is identical, except that a penultimate Galβ1,3GlcNAc is produced. A novel linker will be employed together with glycosyl phosphate (55) and trichloro-acetimidate (56) building blocks. The finished oligosaccharide will be liberated from the solid support using olefin metathesis or ozonolysis.

Approach 2: Preparation of small carbohydrate libraries (Figure 9, next page). H-1 and H-2 tetrasaccharide **10** or closely related oligosaccharide structures will serve as lead compounds for the design and synthesis of oligosaccharide libraries. The different members of the library will be assembled in a spatially separated parallel synthesis strategy such that only one specific carbohydrate is produced in each vessel, thus allowing for ready identification of the desired products. A flexible protecting group ensemble will give rise to a variety of analogs of the H-1 and H-2 tetrasaccharide by modification of the hydroxyl functionalities after selective deprotection of orthogonal protecting groups. Support-bound monosaccharide **3** after removal of the sialyl protecting group will serve as glycosyl acceptor in the reaction with donor **4** to yield disaccharide **5**. Alternatively, coupling with donor **13** will yield the β1,3-linked disaccharide **14**. Both disaccharides will serve as glycosyl acceptors in the reaction with the differentially protected galactosyl donor **11** to furnish trisaccharides **12** and **15**. Selective removal of the different protecting groups at the trisaccharide stage will

reveal the hydroxyl moieties that may be glycosylated or alkylated. Oligosaccharides **16-19** constitute some representative structures that will be generated by fucosylation of the H-1 and H-2 core trisaccharide. Deprotection and cleavage from the solid support will reveal the desired oligosaccharides in the same way the H-1 and H-2 oligosaccharide are released. H-1 structures will be given priority, but all combinatorial structures will be tested to assure that we find the simplest structures with the highest activities.

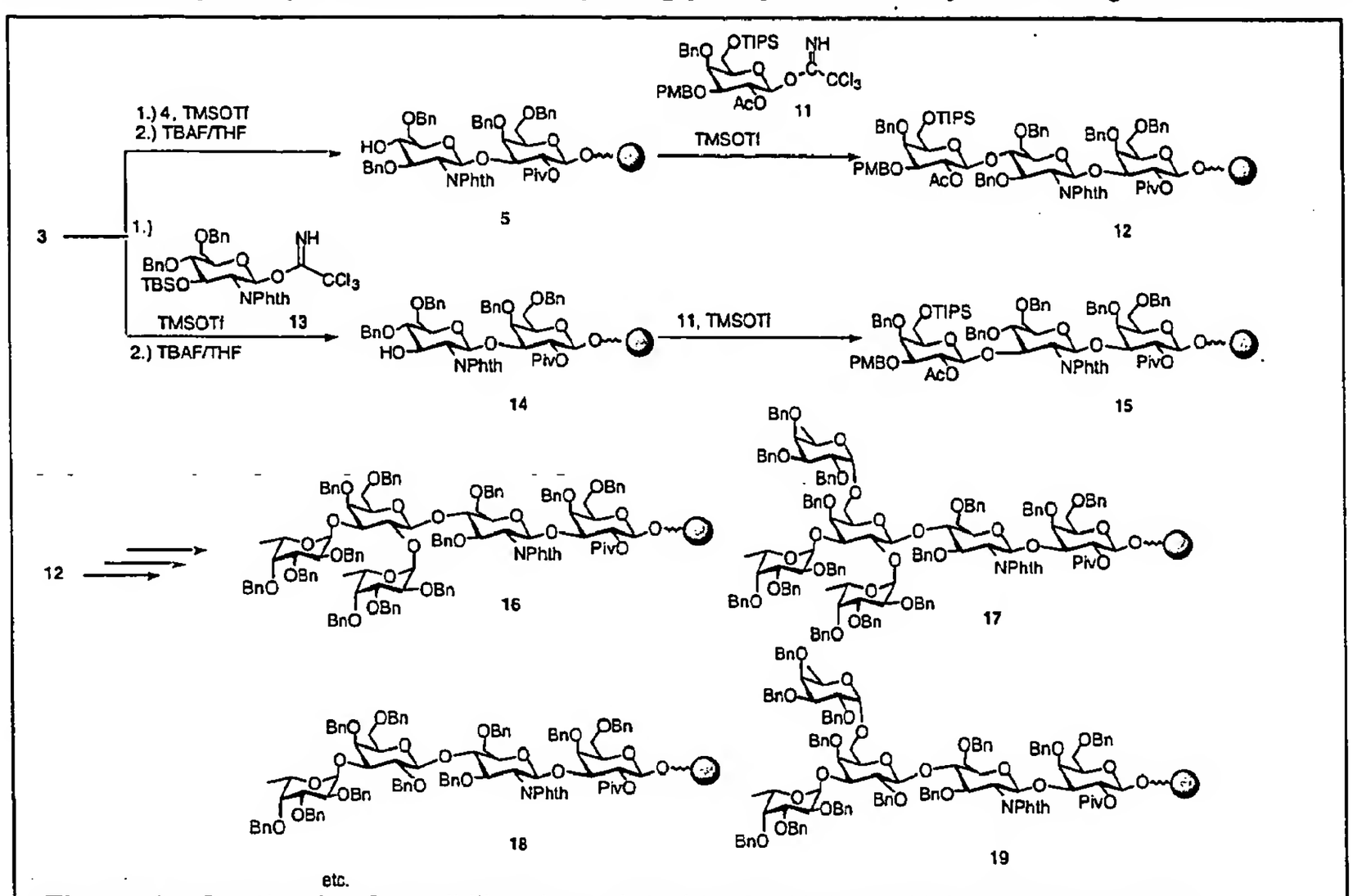


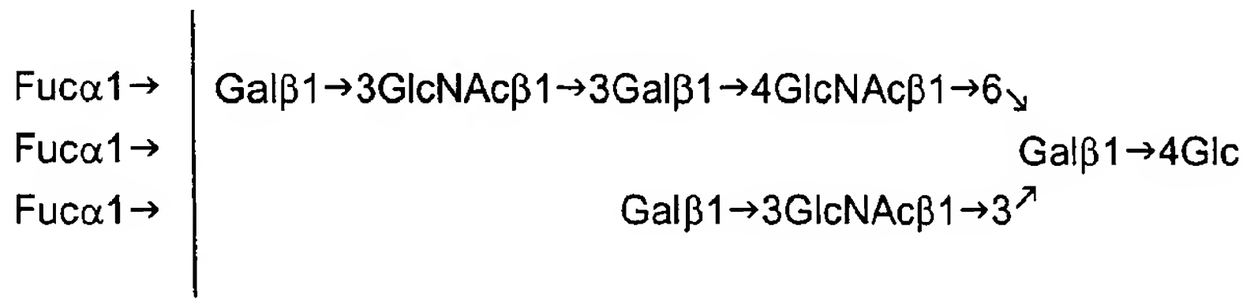
Figure 9. *Synthesis of small libraries related to the H-1 and H-2 tetrasaccharides*

For those oligosaccharides whose synthesis by automated methods is not feasible, we will employ conventional batch process syntheses as described below.

Chemical Synthesis

Criteria for the synthesis of candidate oligosaccharides. The structures chosen as synthetic targets will be based on the putative structure of human milk ST-protective oligosaccharide. Based on the composition determined in the preliminary studies (3 fucose, 4 galactose, 3 *N*-acetylglucosamine, 1 glucose) a molecular weight suggesting a undecaose, and the requirement for binding to *U. europaeus* lectin, a structure such as that of trifucosyl-*iso*-lacto-*N*-octaose (Figure 10) is the candidate for the active fraction. Homologs having fewer or more fucoses and homologs of each arm of the structure are the strongest candidates for active synthetic oligosaccharides.

Figure 10*. Putative structure of the ST-inhibitory oligosaccharide*



The data in Table 6, along with the known characteristics of other biologically important carbohydrate ligands, are consistent with the active part of the structure being the critical determinant in the region of the non-reducing chain termini. This determinant may be displayed on more than one active oligosaccharide. The active determinant probably consists of more than one structural element (each probably a di-, tri-, or tetra-saccharide) situated on different branches. For example, oligosaccharides (**7**) and (**8**) of Scheme 1 could represent the termini of the structure represented in Figure 10. The remainder of the molecule will consist of lactose as a common core at the reducing terminus, probably not contributing significantly to the biological activity. These oligosaccharides, singly or in combinations, will be tested. Together, the structural elements that make up the active determinant will provide a complex surface that docks with the carbohydrate recognition domain of the ST receptor.

When the synthetic compounds have been tested for activity, it may be necessary to design a second series, based on the results. The ultimate aim will be to obtain a compound with full biological activity but as simple a structure as possible, to facilitate the synthesis of amounts adequate for a full set of binding studies.

The structures of the oligosaccharides to be synthesized (Scheme 1) are based on the above criteria.

Scheme 1. Oligosaccharides to be synthesized

(1) Fucα1→3GlcNAc

(2) Fucα1→4GlcNAc

(3) Fucα1→3↘
GlcNAc
Galβ1→4↗

(4) Fucα1→4↘
GlcNAc
Galβ1→3↗

(5) Fucα1→6↘
GlcNAc
Galβ1→4↗

(6) Fucα1→6↘
GlcNAc
Galβ1→3↗

(7) Fucα1→2Galβ1→3GlcNAc

(8) Fucα1→2Galβ1→4GlcNAc

(9) Fucα1→3Galβ1→4GlcNAc

(10) Fucα1→6Galβ1→4GlcNAc

Oligosaccharide synthesis--experimental approach. The choice of glycosyl donors and acceptors, protective group strategies, and coupling conditions for formation of α-L-fucosyl (*cis*), and β-D-galactopyranosyl (*trans*) linkages will be according to the established methods of modern synthetic carbohydrate chemistry (57-59). The reactions will be "conventional" in the sense that the objective is to obtain the target compounds as quickly as possible in reasonable yield, rather than to explore novel chemistry.

Glycosyl donor for α-L-fucosyl residue. The critical requirement is for a donor with a "non-participating" group at O-2 (60). For this purpose 2,3,4-tri-O-benzyl-α-L-fucopyranosyl bromide will be employed, under conditions of halide ion catalysis, a method that has been successfully employed in many syntheses of α-L-fucosyl derivatives (61-65). The use of benzyl ether groups as persistent protective groups (66,67) will have the additional advantage that after coupling, deprotection of donor and acceptor residues in the target compounds can be achieved at the same time by catalytic hydrogenolysis. The use of alternative donors, such as 1-thio, fluoride, trichloroacetimidate, or 4-pentenyl glycosides (68) will only be considered if the above approach is found to be unsatisfactory.

2-Acetamido-2-deoxy-D-glucopyranose acceptors (GlcNAc acceptors). Initially, the compounds will be benzyl, 4,6-benzylidene, or allyl ether derivatives of benzyl glycosides (for details and references, see Scheme 2), but the groups will be manipulated so that primarily benzyl ethers will remain at the end of the synthesis. A final step of catalytic hydrogenolysis will deprotect residues derived from both acceptor and fucosyl donor. The regioselectivity of the reductive ring opening of benzyl 2-acetamido-4,6-O-benzylidene-2-deoxy-D-glucopyranoside derivatives with lithium aluminum hydride/aluminum chloride is dependant on the steric bulk of the substituent at O-3 (69), so in the synthesis of (**6**) (Scheme 1) the desired 4-O-benzyl derivative will inevitably be accompanied by some 6-O-benzyl compound. A chromatographic separation will be necessary, followed by methylation analysis to identify the required material.

β-D-Galactopyranosyl donor (for galactose residues at non-reducing terminus). The primary requirement is for a "participating" group at O-2 (59). Therefore, 2,3,4,6-tetra-O-acetyl-α-D-galactopyranosyl bromide will be employed. Glycosidation will employ silver triflate as promoter, in the presence of acid scavengers (collidine or tetramethylurea), and molecular sieves, i.e., standard conditions for *trans* glycoside coupling (70-72). Should this prove unsatisfactory in any particular case (e.g., because of a lack of strict stereospecificity) Helferich conditions (73) (mercuric cyanide/mercuric bromide promoter) or Koenigs-Knorr conditions (74)

(silver carbonate as insoluble catalyst) can be substituted. Whatever method is used, after chromatographic purification of the product of the first glycosidation reaction, it may be necessary to perbenzylate to avoid any risk of intermolecular acetyl migration during the subsequent fucosylation step. However, if experimentation indicates that this is not a problem, the perbenzylation step will be left out.

The order of glycosidation of the *N*-acetylglucosamine acceptor in the branched trisaccharides **(3)**, **(4)**, **(5)**, and **(6)** will be galactose first and fucose second, because the α-L-fucosyl linkage will be much more unstable to any acidic conditions encountered during coupling reactions, processing, or deprotection steps.

Internal galactose residue in linear trisaccharides. This will be more challenging in terms of strict protective group requirements, especially for compound **(9)** that contains the α-L-fucosyl-(1→3)-D-galactose unit. At O-1 a temporary protective group must allow introduction of a halogen atom as a leaving group in the glycosidation reaction. This should be bromine whenever possible because of its greater reactivity (59), and the introduction should be under the least harsh conditions that are practical. Therefore the O-1 substituent will normally be p-nitrobenzoyl, to be reacted with hydrogen bromide in dichloromethane (75). For compound **(9)**, the acid lability of the protective group at O-3 will not allow this reaction, so a glycosyl chloride, which can be introduced under neutral conditions (67), must be employed instead. At O-2, a "participating" group is necessary for 1,2-*trans* -glycoside formation. Generally, O-benzoyl is favored over O-acetyl because the O-benzoyl group is less labile to basic conditions, less prone to migration, less likely to undergo unwanted ortho ester formation during coupling reactions, and easier to introduce selectively (66). At the position where linkage is desired (O-2 in **(7)** and **(8)**, O-3 in **(9)**, and O-6 in **(10)** a temporary protective group is necessary, that can be removed after the first glycosidation without affecting other linkages or groups. For oligosaccharides **(7)** and **(8)**, the benzoyl group will perform both functions. For oligosaccharide **(9)**, *tert*-butyldimethylsilyl (TBDMS) or tetrahydropyranyl (THP) groups will be used (67). For oligosaccharide **(10)**, we will be able to use a benzoyl group at both O-2 and O-6 because, after coupling and debenzoylation, primary alcoholic O-6 may be fucosylated selectively in the presence of unprotected O-2. Finally, for all the oligosaccharides (except O-2' in **(10)**), the remaining two positions must be occupied by persistent groups (benzyl), to be removed only at the end of the synthesis.

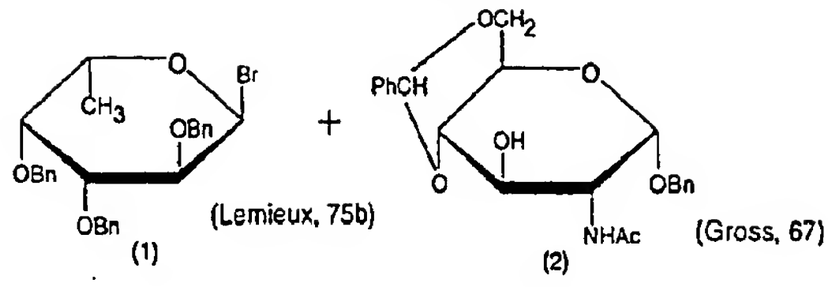
The coupling reaction between the specially protected "internal" galactosyl donor and the protected glucosamine acceptor will employ similar conditions to those described above for the reactions with tetra-O-acetyl-α-D-galactopyranosyl bromide, with special care to avoid the development of acidic conditions in the case of the donor with the acid-labile TBDMS or THP groups. In this case, even with silver triflate as promoter, it will be necessary to tolerate low yields in the reaction of the glycosyl chloride and the poorly reactive OH-4 of the glucosamine compound leading to oligosaccharide **(9)**.

Starting compounds, chromatography, deprotection, and structure confirmation. All the starting materials are accessible from L-fucose, D-galactose, and 2-acetamido-2-deoxy-D-glucose, via procedures in the literature (for details and references, see Scheme 2). Their preparation, and the coupling reactions, will be followed by TLC, and the reaction mixtures will be processed by standard procedures. The products of the coupling reactions will be purified by silica gel column and preparative layer chromatography. Deprotection steps are expected to be straightforward as a result of careful choice of the groups to be used. Alkaline conditions will be strictly avoided whenever a 1,3 linkage is present and a compound is unsubstituted (by a protective group or another sugar residue) at O-1. Purity of final products will be determined by TLC and HPLC, and a final purification by passage through a column of Bio-gel P-2 and/or a coupled column of cation and anion-exchange resins will be performed when necessary. Structures of key intermediates and final products will be confirmed by permethylation analysis and mass-spectrometry.

In Scheme 2 (next two pages), the format for the references is (first author, date).

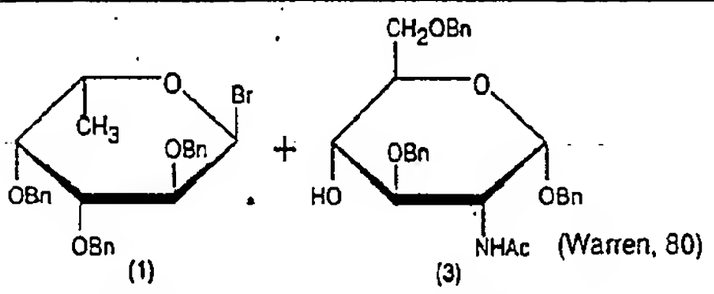
Scheme 2

1.



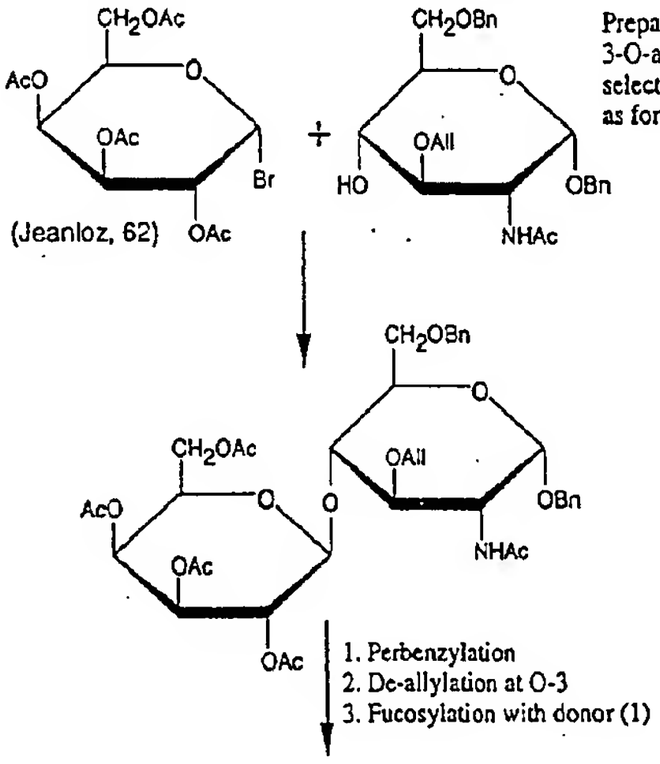
(1) (Lemieux, 75b) (2) (Gross, 67)

2.



(1) (3) (Warren, 80)

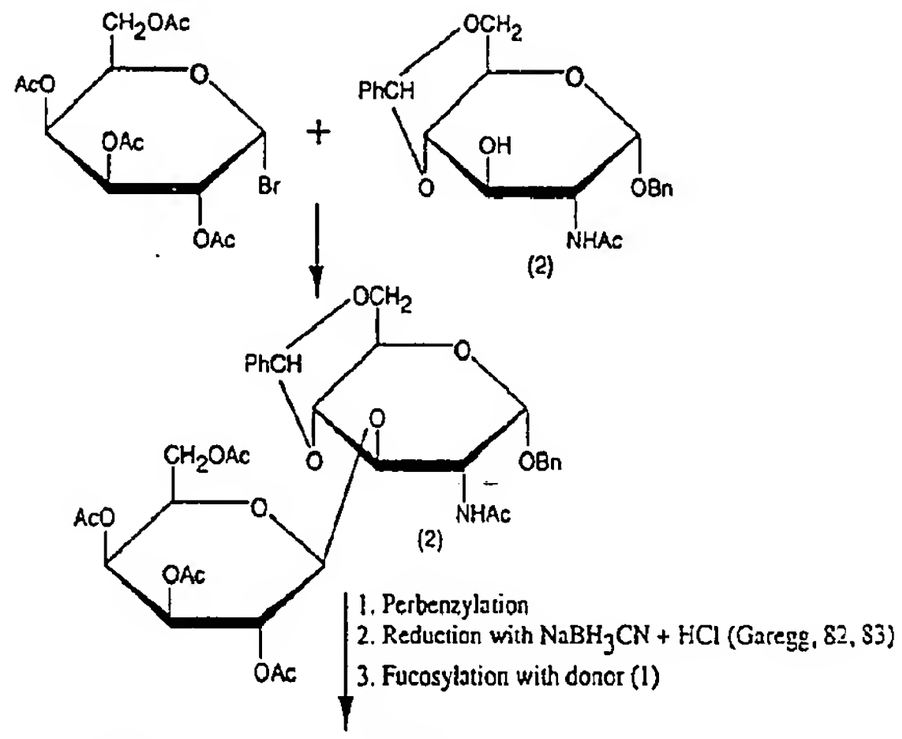
3.



(Jeanloz, 62)

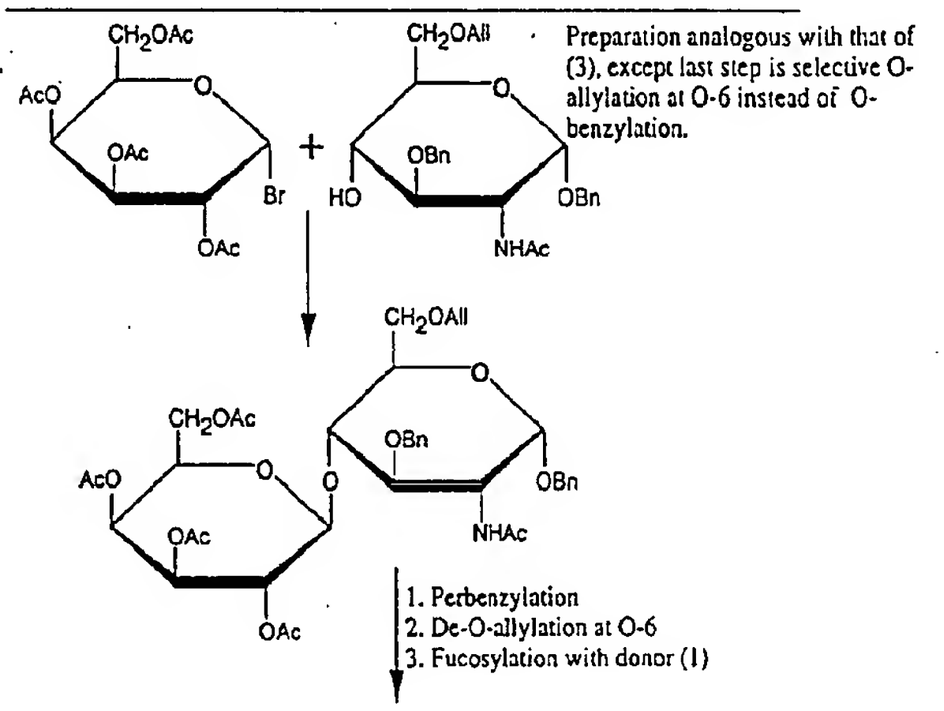
Prepared from (2) in 3 steps: 3-O-allylation, hydrolysis, and selective O-benzylation at O-6, as for preparation of (3).

4.



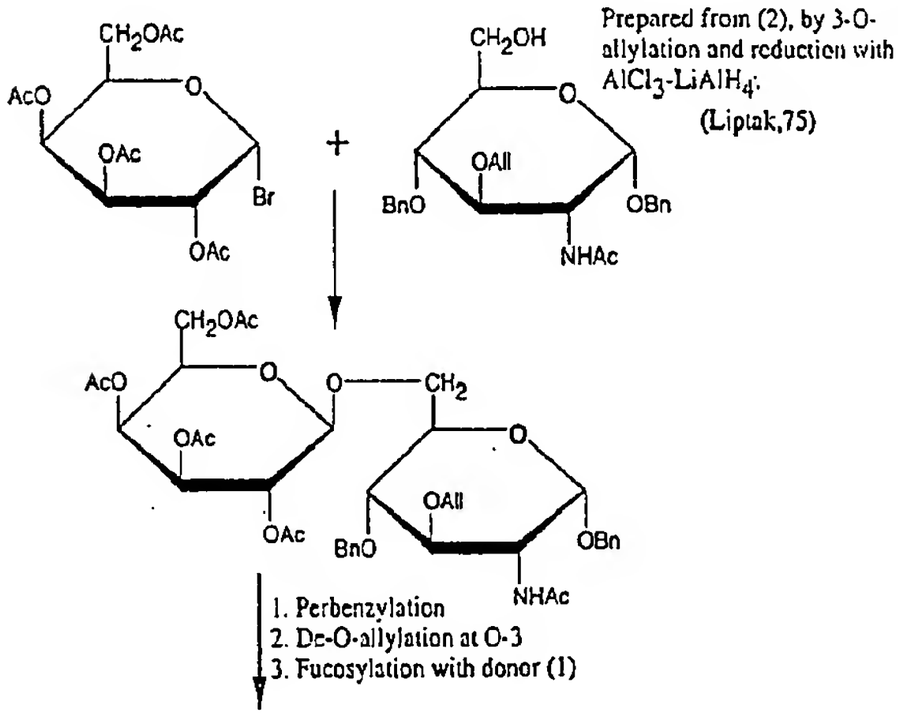
(2)

5.



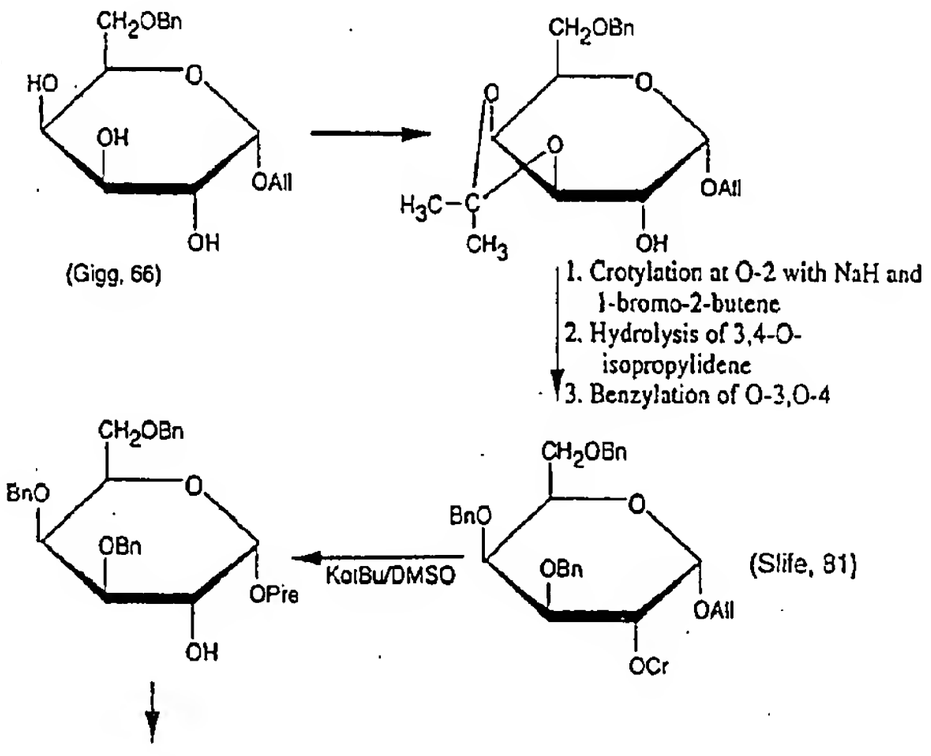
Preparation analogous with that of (3), except last step is selective O-allylation at O-6 instead of O-benzylation.

6.



Prepared from (2), by 3-O-allylation and reduction with $AlCl_3$-$LiAlH_4$: (Liptak,75)

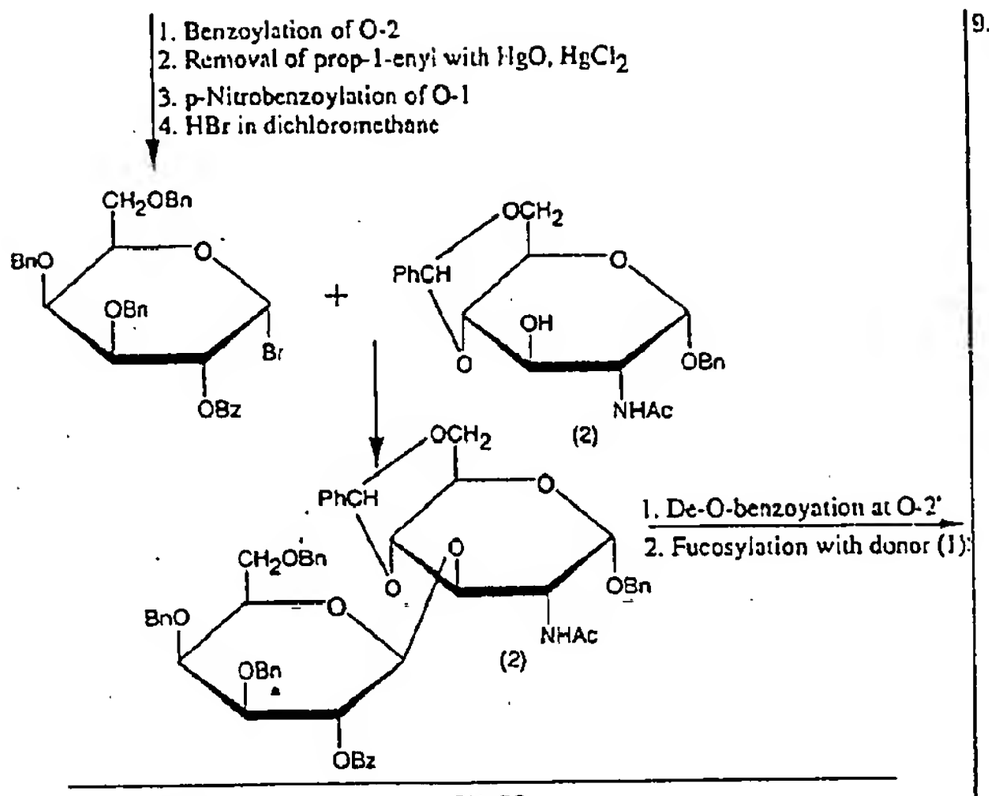
7.



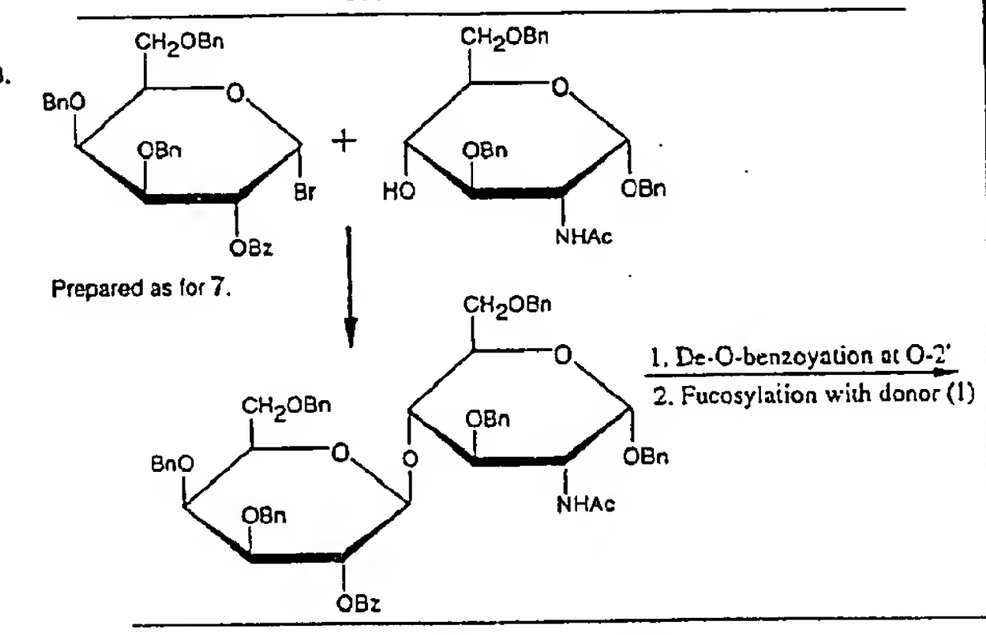
(Gigg, 66) (Slife, 81)

(continued on next page)

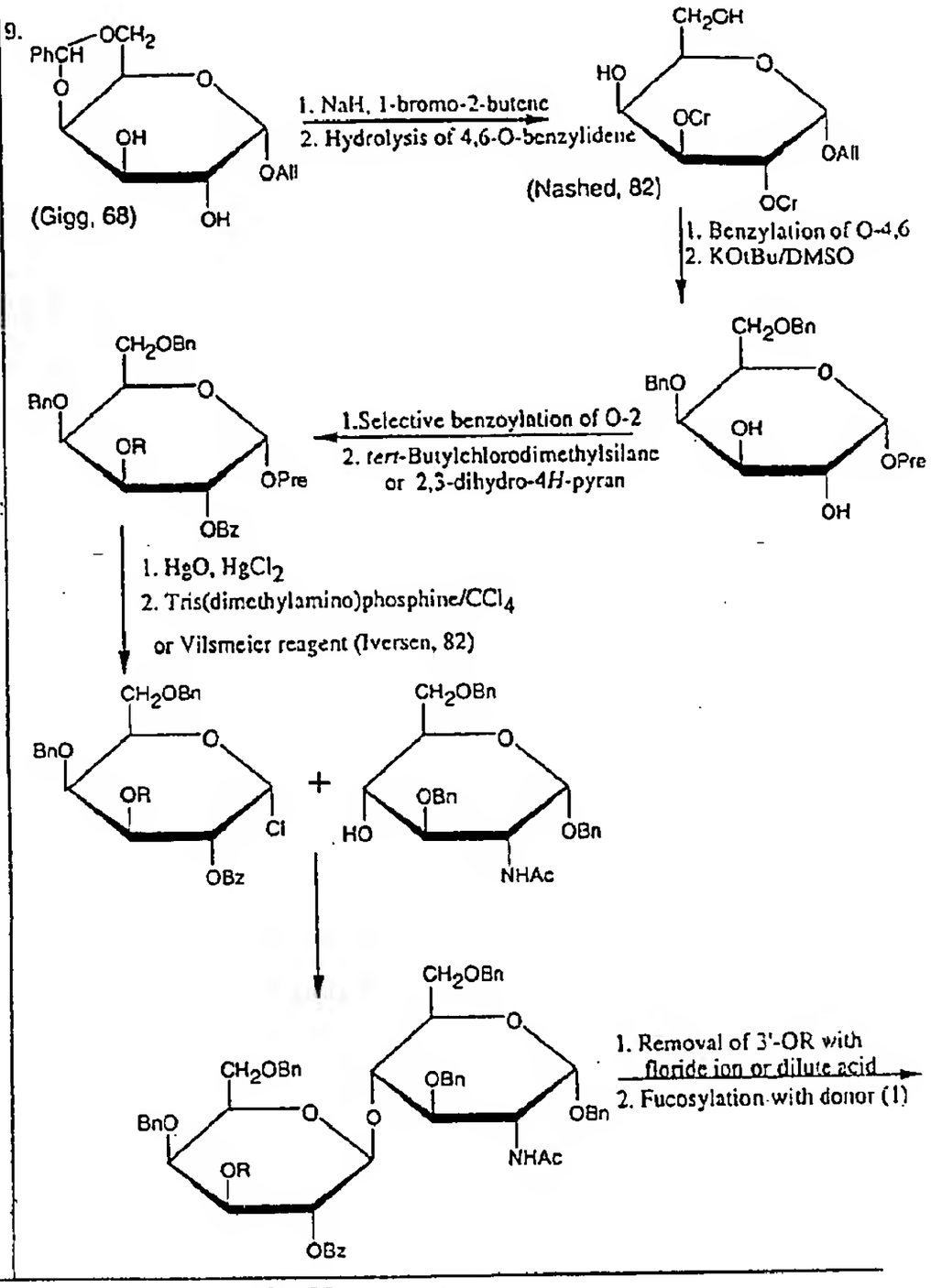
(7. continued)



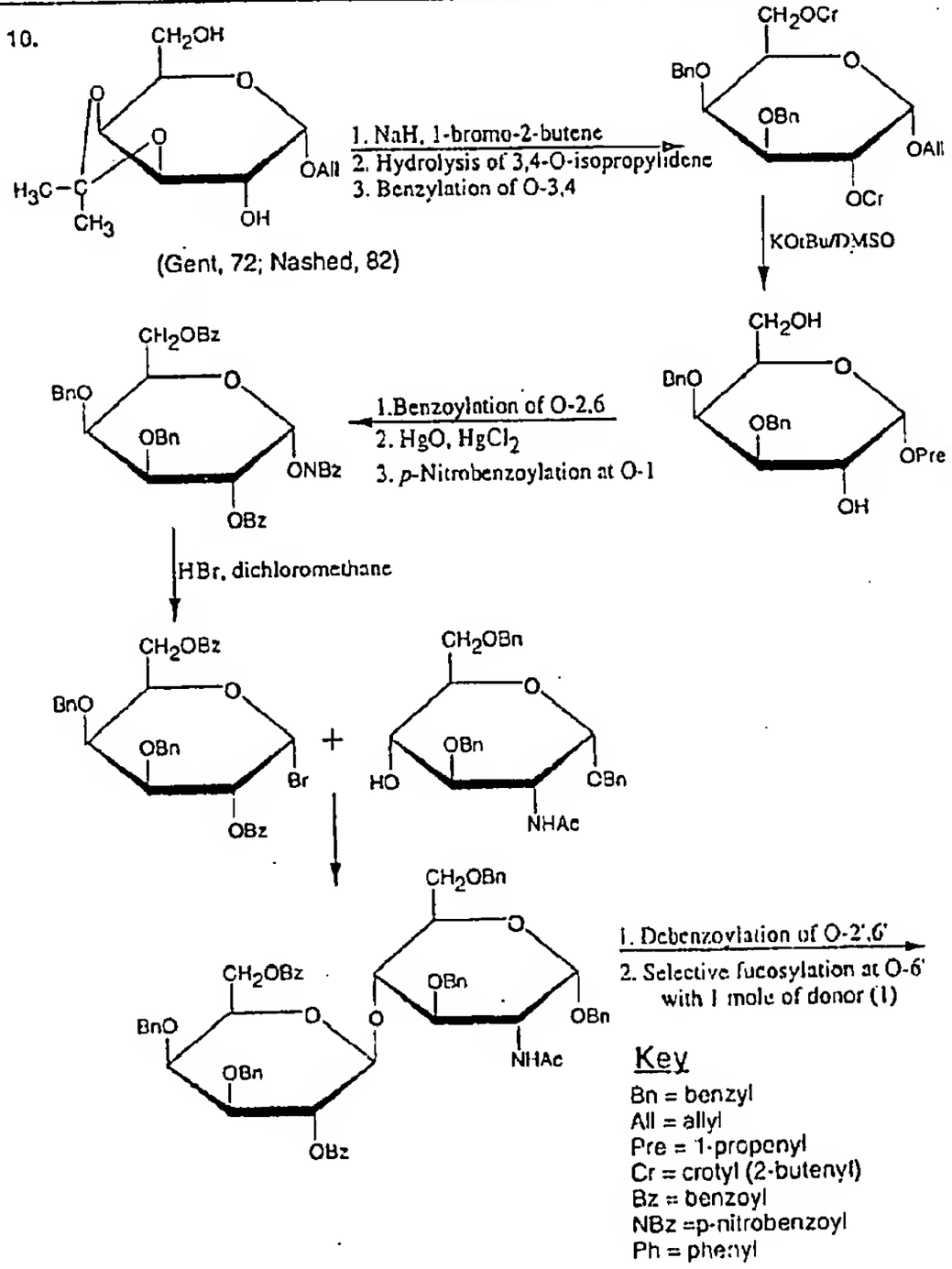
8.



9.



10.



Key

Bn = benzyl
All = allyl
Pre = 1-propenyl
Cr = crotyl (2-butenyl)
Bz = benzoyl
NBz =p-nitrobenzoyl
Ph = phenyl

Detailed experimental plans for each target oligosaccharide are summarized in Scheme 2. The GlcNAc acceptors are shown as benzyl glycosides for simplicity, though 2-bromoethyl glycosides or 8-methoxycarbonyloctyl glycosides will be substituted if necessary for protein conjugation (see Order of Synthetic Experiments, below). Scheme 1 oligosaccharides **(3)** (76), **(4)**(77), **(6)** (62), **(7)** (78) and **(8)** (64) have been synthesized before, and those experimental and spectroscopic data will be useful for the preparation of intermediates and characterization of our products.

Order of synthetic experiments. In Scheme 2, the starting compounds (1) and (2) for synthesis of target oligosaccharide **(1)** can be prepared quickly because a small number of steps is involved. Oligosaccharide **(1)** will be a test compound for the conversion into the *p*-nitrophenyl glycoside, en route to the *p*-aminophenyl glycoside. If the α-L-fucosyl linkage in **(1)** survives these steps, the remaining syntheses, of **(2)** to **(10)**, will be performed as shown in Scheme 2 as described above.

Compositional and Structural Analyses

Microanalysis of sugar ratios by GC (79). The sample is transferred into a capillary tube (1 mm i.d. x 35 mm) in aqueous methanol (50%). The solvent is removed during centrifugation under vacuum. The sample is dried in a vacuum desiccator over P_2O_5. Dry methanolic HCl (0.75 mol/L; 25 mL) and methyl acetate (5 mL) are added before the tops of the tubes are resealed in a flame. The tubes are incubated at 80˚C for 2 hand allowed to cool to ambient temperature, whereupon the top of the tube is scored and cracked open. The tube is placed under vacuum with centrifugation to remove the methanolic HCl. Internal standard (methyl heptadecanoate, 2 nmol in 5 ml methanol) is added, and the solvent is removed by vacuum centrifugation. Freshly made 50% acetic anhydride in dry pyridine (5 mL) is added, the tubes are resealed, and the acetylation allowed to proceed for 14 h at ambient temperature (the reaction is complete after 2 h). The top of the tube is scored and cracked open, whereupon an aliquot (1 μL) of the contents are injected into a gas chromatograph fitted with a 30-m DB-1 column. Peaks are detected by flame ionization. After injection, the temperature is held at 150˚C for 15 min and then raised by 4˚C per min to a maximum temperature of 300˚C. Peak areas are calculated with an HP integrator. This method gives results that are suitable for determining both sugar ratios of a pure compound and absolute quantitation of sugars in a sample. This method yields consistently good results with approximately 1 μg (1 nmol) of oligosaccharide.

Mass spectrometry. All synthetic products will be analyzed by mass spectrometry to assess purity and confirm structures. The number of components in a sample and their molecular weights are determined by matrix-assisted laser desorption ionization mass spectrometry. MS/MS of peracetylated sample is used to obtain compositional information on the individual components of a mixed sample. The fragmentation pattern in the fast atom bombardment mass spectrum gives some insight into the structure of a pure sample. MS/MS of derivatives can be used to obtain complete structural information even for a sample that contains a major component in the presence of appreciable impurities. Linkage of pure compounds is established by GC/MS analysis of partially O-methylated hexitols and hexosaminitol acetate (PMAAs) (80). These analyses will be performed by the Mass Spectrometry Resource Center, Boston University; see letter by Dr. Catherine Costello.

Potential difficulties, limitations, and alternative approaches. Two alternative approaches toward the synthesis of these oligosaccharides are proposed. We anticipate that some oligosaccharides are probably more readily synthesized by automated techniques, while others, such as those containing amino sugars, might be most readily synthesized by classical synthetic techniques. If both approaches fail to produce a given structure, that structure or its closest homolog can be isolated from human milk. This third approach is intrinsically more expensive. No technical difficulties are anticipated for the analysis of these oligosaccharides as the methods proposed were developed in our laboratory and are in routine use. However, we anticipate yet another method of analysis as our laboratory develops an LC/MS method for oligosaccharide analysis.

Specific Aim 2. Test each of these trisaccharides and tetrasaccharides, individually and in combination, for their ability to inhibit ST-induced diarrhea in the suckling mouse and compare their activity with that of the human milk oligosaccharide fraction

Logarithmic dilutions of the synthetic and natural protective fucosyloligosaccharides will be tested in the suckling mouse model described below. The resulting data will give us an approximate range of concentrations at which the test compounds are inhibitory. This range will be refined, and the precise minimum inhibitory dose determined, by assays using incremental dilutions of the compound (1:1, 1:2, 1:3, etc.). Regression analysis for each test compound or mixture will define the minimum inhibitory dose.
We hypothesize that some components of the fucosyloligosaccharide library will have activity against ST due to homology with a critical motif in the native human milk oligosaccharide that inhibits ST. Furthermore, we anticipate that inhibitory oligosaccharides will exhibit synergy when tested as mixtures. Thus, we will test these fucosyloligosaccharides, singly and in combination, to try to maximize the efficacy of ST inhibition. The activity of this mixture will be compared with the activity of the human milk fucosyloligosaccharide fraction. This will allow us to determine the extent that the anticipated lower activity of the simpler molecules can be offset by using them at higher doses.

Suckling mouse assay. This assay has been successfully used by our laboratory for many years. Inbred C57BL/6J mice born in our facility are used to avoid the experiment-to-experiment variation that occurs when timed-pregnant outbred mice from commercial sources are used. On the evening before the experimental procedures, a custom-built environmental unit is sterilized with alcohol. Three-day-old suckling mice (day of birth is day 0) are separated from their dams. Each litter is evenly distributed by systematic allocation into cages marked by experimental group, with a minimum of 10 pups per group. Cages are kept overnight on the temperature- and humidity-controlled environmental unit (34°C±2°C, relative humidity 95%±4%) designed to replicate conditions in their dams' nests. This procedure minimizes any chance of hypothermia and/or dehydration that would affect the dependent variable, which is death mediated by diarrhea-induced dehydration. The next morning, ST (4.5 units/pup) is administered by orogastric intubation along with oligosaccharide(s) in a total volume of 0.03 mL (30 μL) of isotonic PBS. Mice are observed hourly for 8 hours.

The number of pups surviving at 8 hours after toxin administration reflects the efficacy of protection by oligosaccharide. Each oligosaccharide group will be compared to a saline control group, and significance of outcomes tested using Fisher's Exact Test with a Bonferroni adjustment to compensate for to multiple statistical comparisons. Survivorship in the saline group is typically 20% (4 of 20 pups). Setting α=.05, power>.70, a sample size of 20 mice/group is adequate to detect differences in survivorship of at least 67% (14 or more out of 20) in the oligosaccharide test group. These estimates are based on the preliminary data obtained under an animal research protocol at the Shriver Center, active from 1985-2000. The suckling mouse assay requires approximately 15 3-day-old pups per group, and 70-80 pups, enough for positive and negative control groups and three experimental groups, can be available approximately two times per month. We estimate between 45 and 60 mice will ultimately be required for each dose of each test compound. Thus, we estimate that these comparisons of minimum inhibitory dose will require 3 years of testing.

Potential difficulties, limitations, and alternative approaches. The proposed suckling mouse assay has been in use by our group for over 20 years, and we do not anticipate any technical difficulty with this assay. If none of the proposed oligosaccharides shows protective activity at the concentration in milk, we would keep testing the most promising at progressively higher doses until we find a level that results in measurable protection. Concurrently, we would synthesize and test larger homologs of these oligosaccharides until we find the optimum combination of ease of synthesis and efficacy. This will allow an evaluation of the potential clinical utility of these synthetic oligosaccharides.

Specific Aim 3. Investigate the mechanism of ST inhibition by synthetic and natural milk oligosaccharides in a human enterocyte cell culture system of T84 cells and in rat intestine

The suckling mouse assay, while a highly relevant and sensitive in vivo model of ST-induced diarrhea in humans, does not address the precise mechanisms of the oligosaccharide-mediated protection against ST. T84 cells, a line of human enterocytes, are commonly used to study the mechanism of ST activity. Using ST-treated T84 cells, we have found evidence suggesting that the protective fucosyloligosaccharide of human milk binds to the extracellular domain of the guanylate cyclase, preventing guanylate cyclase binding and activation by ST (45). Similar studies must be conducted for synthetic oligosaccharide mixtures and pure oligosaccharides that are found to be active in vivo (Specific Aim 2). If the mechanism is the same in all cases, the different strengths of inhibition by the compounds will indicate relationships between structural elements and function. This information will be helpful in finding the smallest most active structure.

We will also determine whether TF*i*LNO (trifucosylated-*iso*-lacto-*N*-octaose) inhibits the binding of guanylate cyclase by guanylin, its endogenous ligand. Guanylin is the physiologic regulator of fluid and electrolyte balance in the intestine (54); by stimulating intestinal guanylate cyclase, it moves fluid into the lumen. Because breastfeeding infants ingest a large quantity of oligosaccharides that are not digested or absorbed, their chyme has high osmolarity, which tends to draw fluid into the lumen, leading to loose stools and, potentially, osmotic diarrhea. TF*i*LNO and other fucosyloligosaccharides may help breastfeeding infants maintain optimal fluid and electrolyte balance by inhibiting guanylin. This hypothesis will be tested by determining the effect of milk oligosaccharides on guanylin-induced activation of guanylate cyclase in T84 cells and binding of guanylin to T84 cell membranes.

We will use a rat intestinal loop model to test our supposition that ST-associated diarrhea and death result from an ST-induced increase in fluid and electrolyte transport into the gut. If the same fucosyloligosaccharides that inhibit ST-induced death due to diarrhea in the suckling mouse and inhibit guanylate cyclase activity in vitro also inhibit ST- or guanylin-induced fluid flux in vivo in closed rat jejunal loops, this will strongly support the conclusion that these are all facets of the same mechanism of inhibition.

We propose to use the in vitro T84 model and in vivo rat intestinal loop model to address the following:
a) Do the synthetic and human-milk-derived fucosyloligosaccharides act by the same mechanism?
b) Is the mechanism identical to that of trifucosylated TF*i*LNO?
c) Is guanylin activity, which is a major contributor to fluid and electrolyte homeostasis in the gut, affected by these fucosyloligosaccharides?
d) Do the same oligosaccharides that inhibit ST and guanylin activity in vitro also inhibit their effect on fluid flux to the lumen of the gut in vivo?

We have demonstrated that the fucosyloligosaccharide fraction of human milk, which is known to contain hundreds of components, prevents ST-induced activation of guanylate cyclase in T84 cells and binding of radioiodinated ST ([^{125}I]-ST) to T84 cell membranes (45). Our current understanding is that this inhibition is due to a single component (TF*i*LNO), but it may be modulated by other components of the mixture.

The synthetic fucosyloligosaccharide combinations produced in Specific Aim 1 that are active in suckling mice (Specific Aim 2) will be tested for their ability to inhibit ST binding and guanylate cyclase activation in T84 cells. In the presence of a range of oligosaccharide concentrations encompassing those found in human milk, T84 cells will be treated with 0.5 µM ST (a maximal dose) and guanylate cyclase activity will be measured. The ability of these oligosaccharide fractions to inhibit specific binding of [^{125}I]-ST to T84 cell membranes will be determined. If inhibition is always by the same mechanism (our favored hypothesis), the relative strength of inhibition can be used to relate specific structural motifs to function. If, however, the mechanisms of action differ; for instance if milk oligosaccharides nonspecifically inhibit the intracellular domain of guanylate cyclase, we may conclude that they contain multiple functional molecules that act in complementary or synergistic ways. Either way, the data can be used to design further oligosaccharides to be tested for optimum activity. Similar experiments with natural and synthetic pure TF*i*LNO will be performed to compare their inhibitory activity with that of the mixtures. These results may confirm that it is *TFi*LNO *alone* that is protective; conversely, if they reveal that *TFi*LNO requires the presence of other milk oligosaccharides for full activity, we can use this information to define optimum synergistic mixtures.

We will measure guanylate cyclase activation by guanylin by incubating T84 cells with this concentration of guanylin, (10^{-8}M to 10^{-4}M) to determine the optimal concentration for maximum activation of the enzyme. Cells will be incubated with guanylin in the presence of a range of concentrations of the total milk oligosaccharide fraction, and inhibition of guanylate cyclase activity will be measured. ST will serve as a positive control in this experiment, and we will also determine if the oligosaccharides alone affect basal guanylate cyclase activity.

Guanylin will be radiolabeled with Iodine-125 ([^{125}I]). Biological activity of the labeled material will be determined by guanylate cyclase activation in T84 cells. To determine if guanylin binds to the same moiety on the receptor as does ST, we will perform binding experiments with [^{125}I]-guanylin in the presence of a range of concentrations of native ST, with molar ratios ranging from 0.01 to 100x that of guanylin. Since ST is known to have greater avidity for its receptor than guanylin, this strategy is more likely to yield results than if we were to use [^{125}I]-ST with native guanylin.

The ability of the total oligosaccharide preparation to inhibit guanylin binding will be measured by incubating T84 cell membranes with [^{125}I]-guanylin in the presence of a range of concentrations of the total human milk oligosaccharides as described for ST (above). If, as we hypothesize, the preliminary experiments prove that the total milk fucosyloligosaccharide preparation proves to be an effective inhibitor of guanylin, we will repeat the experiments described for ST using the synthetic oligosaccharide combinations to inhibit guanylin binding and activation of guanylate cyclase by guanylin. Oligosaccharide concentrations will include those found in human milk and those that inhibit guanylate cyclase in ST-stimulated cells. The ability of the oligosaccharides to inhibit basal guanylate cyclase activity in T84 cells without guanylin or ST will be measured. These experiments will confirm that ST and guanylin act by the same mechanism, or they will provide information about different mechanisms that may be involved.

To demonstrate that the in vitro inhibition of ST- and guanylin-induced changes of guanylate cyclase result in diarrhea through changes in intestinal fluid transport in vivo, we will measure fluid accretion in rat jejunum using the closed loop method of Ieda et al. (81). Jejunal loops will be inoculated with 10^{-7}M ST or 10^{-6}M guanylin in 2 mL saline or saline alone; after 10 to 30 min, fluid accumulation will be measured by weighing the loops before and after they are drained. Inhibition of fluid accumulation by total milk oligosaccharides or synthetic oligosaccharide mixtures will be determined by pretreating the loops with oligosaccharide solution 5 min before adding ST or guanylin and by including the oligosaccharide(s) in the test mixture. Oligosaccharides will also be tested in the absence of ST or guanylin to see if they affect fluid transport. Fluid transport will be calculated as mL fluid/30 min/g wet weight of jejunal segment. Any nonspecific fluid loss is controlled for by normalizing data to a fixed amount of polyethylene glycol (PEG 4000), a nonabsorbable marker, in the test solution. Recovery of PEG from the jejunal loop will be quantified by the turbidimetric method of Malawer et al. (82).

These jejunal loop experiments should provide basic information about the role of human milk oligosaccharides in regulating normal fluid and electrolyte homeostasis in the infant intestine; they should also confirm the mechanism by which the oligosaccharide prevents disruption this homeostasis by ST. This information is crucial if milk oligosaccharides are to be developed as a possible therapy for diarrhea.

Specific Methods

Cell culture. T84 cells at passages 52 to 53 are grown in T75 flasks in DMEM-F12 medium containing 10% newborn calf serum as described previously (45,83,84). For experiments in intact cells, harvested cells are plated in 24-well tissue culture plates and allowed to grow to confluency over 7 days prior to use. T84 membranes from cultures grown at least 7 days in 10-cm diameter dishes by are prepared being scraped on ice in hypotonic buffer, homogenized, and centrifuged, as described previously (45,83,84).

Guanylate cyclase assay and cGMP measurement. Guanylate cyclase activity is measured by determining cyclic guanosylmonophosphate (cGMP) levels in cells per unit time. For experiments measuring cGMP accumulation in intact T84 cells, the usual medium is replaced with 0.5 mL of serum-free medium containing

1mM of the phosphodiesterase inhibitor isobutylmethylxanthine. If used, oligosaccharides are added first, then 0.5 µM ST or guanylin at the concentrations described above; cGMP accumulation is allowed to proceed for 60 min at 37°C prior to termination with 0.5 mL of 0.2M HCl. Cyclic GMP is measured by a specific radioimmunoassay.

Radiolabeling of ST and guanylin and binding assays. ST and guanylin are labeled by the lactoperoxidase oxidation method and purified as previously described (85). Binding assays, performed according to Crane et al. (83,84), are carried out at 30°C for 60 min at pH 5.8 and are terminated by filtration on glass fiber filters and extensive washing. The specificity of binding will be determined by extensive washing of membranes containing bound [^{125}I]-guanylin on a filter with medium alone, or with medium containing a 100-fold molar excess of native (unlabeled) guanylin. Binding in the presence of TF*i*LNO and Scatchard analysis of the binding data will allow its inhibition constant (K_i) to be determined, its binding constant to guanylate cyclase to be measured, and the binding site to be characterized. The mechanism of inhibition can then be studied at the molecular level.

Rat closed jejunal loop assay. Male Wistar rats (230±30 g) will be kept under standardized conditions (23°C, 50-55% humidity, artificial lighting between 09.00 and 21.00 h) for 7 days and deprived of food but not water for 24 h before the start of the assay. The animals will be anesthetized by intramuscular injection of ketamine/xylazine (80 mg/kg ketamine, 4 mg/kg xylazine) then placed on a heated pad under an overhead lamp, keeping their rectal temperature at 36-38°C. The abdomen will be opened and the jejunum ligated, making the first ligation 5 cm distal to the flexura duodeni jejunalis and the second 25 cm distal to the first. Luminal debris will be removed by washing with 20 mL of 154 mM saline solution at 37°C followed by air, and gentle manipulation of the loop. The loop will be returned to the abdominal cavity for 30 min, then experiment will be started by filling the loop with the test solution containing PEG 4000. Following a 10-30 min. absorptive period, the animals will be euthanized by carbon dioxide gas and the jejunal loop excised (86).

Potential difficulties, limitations, and alternative approaches. We previously used cultured T84 cells to measure ST-induced guanylate cyclase activation. The standard method for measuring guanylin activity is a similar T84 cell assay. Therefore we do not anticipate any difficulty with the cells or the guanylate cyclase assay. However, the iodination of guanylin, like the iodination of any toxin, could potentially alter its activity. Iodination of ST by lactoperoxidase oxidation results in biologically active [^{125}I]-ST. The structure of guanylin is similar to that of ST and contains tyrosine; therefore, the lactoperoxidase technique is likely to work for guanylin. If this results in loss of activity, the Bolton-Hunter technique will be utilized, as it is also a gentle technique. Should both of these fail, we will contract with an experienced supplier of radionuclides for them to iodinate guanylin by even more gentle techniques. For measuring biologic activity, we propose both the T84 assay and the rat intestinal loop assay as complementary techniques, but each can be thought of as an alternative to the other, should one technique not prove useful. Moreover, should the rat intestinal loop assay (a published method of choice) fail, rabbit intestinal loops and mouse intestinal loops might be substituted. These experiments depend on at least one compound showing activity at some concentration; our approach toward finding that compound is outlined in specific aim 2.

Specific Aim 4. Determine the genetic basis of variability in the concentrations of oligosaccharides in human milk that protect against ST

Our epidemiologic and genetic data are from our prospectively studied cohorts (current and previous, see Figure 4 in Core) of mother-infant dyads in San Pedro Martir, Mexico City. Our preliminary results describe the heterogeneous expression of the human milk oligosaccharides. This heterogeneity is strongly associated with differential protection against diarrhea caused by many pathogens, including ST. The heterogeneous expression is also strongly associated with the Lewis blood group phenotype of the lactating mother. Our results suggest that, within a Lewis blood group phenotype, multiple genotypes may express different fucosyloligosaccharide patterns in milk, each with distinctly different capacities for protecting the nursing infant. However, phenotyping for Lewis blood group is imprecise, and the Lewis genotypes are degenerate in that multiple genotypes express as the same phenotype. Therefore, in conjunction with the Glycobiology, Molecular Biology, and Molecular Epidemiology cores, we propose to define the relationship between

maternal Lewis and secretor genotypes and expression of oligosaccharides in milk. Furthermore, the relationship between the Lewis genotype of the infant, oligosaccharide profile of the mother's milk, and risk of ST-associated diarrhea will be determined.

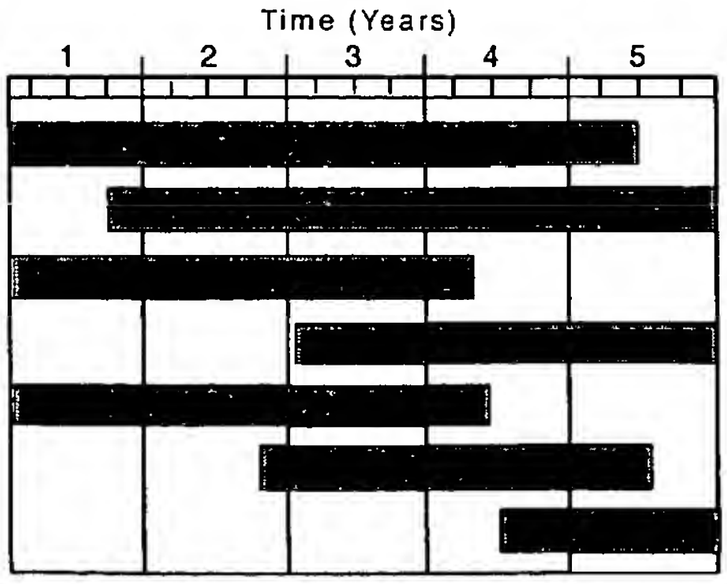
The maternal Lewis genotype will be determined from the DNA of their milk cells by PCR. The infant Lewis genotype will be determined from the DNA in their buccal cells, obtained as swabs. The oligosaccharide profiles of the milk will be measured by HPLC and capillary electrophoresis techniques that we have devised, and are in routine use in our laboratories (see Glycobiology Core). We anticipate that the presence of oligosaccharides in milk will protect against ST-induced diarrhea, as our preliminary data indicates. We also anticipate the particular Lewis genotypes will be associated with higher levels of protective oligosaccharides in milk and with protection of the infant.

To fulfill this aim, we will examine the expression of oligosaccharides in milk in relation to maternal genotype. Specifically, we will describe, among the nine or more distinct secretor and Lewis histo-blood group genotypes, the pattern in (a) the mean maternal milk concentrations of TF*i*LNO or its surrogate, LNF I and (b) the mean value of individual maternal samples of the ratios of milk 2 to 3 linked fucosylated oligosaccharides. For hypothesis testing purposes, we will classify the unique genotypes into 3 genotype groups based on our current understanding of the potential dominance of of the secretor gene in relation to the Lewis gene (see Lewis synthesis pathoway, Figure 6). Using the mean concentration of LNF I as a surrogate measure for TF*i*LNO, and with the available sample size of 415 (see Figure 4, in Core), we anticipate > 90% power to detect significant differences among these three groups, and >80% power to detect significant differences between any 2 groups. We will use analysis of variance and two-sample t-test for comparison of milk values by genotype groups. Correlation analysis will be used to determine the relationship between the milk concentration of TF*i*LNO and the ratio of 2 to 3 linked fucosylated oligosaccharides. The association between concentrations in milk oligosaccharides and ST diarrhea in breast-fed infants will be addressed using the previous cohort (n=286), from which 7 symptomatic ST infections were detected. Milk values will be expressed as concentrations per mL of milk and as a percentage of the total oligosaccharides in the sample; the average concentrations will be compared between the milk of mothers whose children were ST-infected symptomatically (n=7) to those not ST-infected (n=279) while breastfed (see also Core Research Design and ST Project Specific Aims 1 and 2).

Potential difficulties, limitations, and alternative approaches. We do not anticipate technical problems with our analytical methods for neutral (87) or acidic (88) milk oligosaccharides in routine use in our laboratory. However, the throughput for the neutral oligosaccharide method is limiting. Therefore, we have purchased a state of the art LC/MS with an autosampler and diode array detector, and are developing a technique to analyze underivatized neutral oligosaccharides. Should this new technique prove unsatisfactory, we will revise the scope of the study to reduce the number of samples. If chromatographic peaks contain more than one component, each component can be individually measure by dual specific ion monitoring made possible by the quadrapole mass spectrometric detector.

TIMETABLE

Specific Aim	Subject
1	Synthesis of oligosaccharides (syntoligos)
2	Measure ST inhibition by syntoligos in mice
3	Δ Guanylin activity by milk oligosaccharides
3	Δ ST and guanylin activity by syntoligos
4	Aquisition of human milk samples
4	Analysis of oligosaccharides in milk samples
4	Data analysis of milk oligosaccharides



E. Human Subjects Research

The research on human milk in this project qualifies for Exemption 4 according to the University of Massachusetts Medical School IRB (Docket #10432 dated 9/18/01). The proposed research involves laboratory tests on already existing samples collecgted under the Core (see Core section for population sample). The analysis of the oligosaccharides of human milk are performed on specimens for which the information is recorded by the Epidemiology Core in such a manner that subjects cannot be identified, directly or by identifiers linked to the subjects, by the investigators in this project who are blinded during the analysis. The codes are broken by members of the Epidemiology Core.

Inclusion of women. All human milk samples are obtained from women of childbearing age. Women of childbearing potential are not excluded routinely from participation.

Inclusion of minorities. See Core section for the breakdown of minorities for the Cincinnati sites. Milk samples from eastern Massachusetts (Boston/Worcester area) are 20% Black, 5% Hispanic or Latino, and 75% White.

Inclusion of children. See Core section for the breakdown of children from the Cincinnati and Mexico sites.

F. Vertbebrate Animals

MICE

1) The suckling mouse assay utilizes the inbred strain of Mus musculus, C57BNL/6J, of random mixed gender. Two-day-old mice are separated from their dams and evenly distributed by systematic allocation into cages with a minimum of 10 pups per group. Cages are kept overnight in a temperature- and humidity-controlled environmental unit designed to replicate conditions in their dams' nests. This procedure minimizes any chance of hypothermia and/or dehydration that would affect the dependent variable, which is death mediated by diarrhea-induced dehydration. The overnight separation ensures that all pups begin the experiment with empty stomachs for uniformity. The next morning, ST (4.5 units/pup) is administered by orogastric intubation along with oligosaccharide(s) in a total volume of 0.03 mL (30 μL) of isotonic PBS. Mice are observed hourly for 8 hours. The number of pups surviving at 8 hours after toxin administration reflects the efficacy of the protection by the oligosaccharide. Each oligosaccharide fraction group will be compared to a saline control group, and significance of outcomes tested using Fisher's Exact Test with a Bonferroni adjustment to compensate for additive Type I error due to multiple comparisons. Survivorship in the saline group is typically 20% (4 out of 20 pups). Setting α=.05, power >.70, a sample size of 20 mice per group is adequate to detect a difference in survivorship of at least 74.5% (14 or more out of 20) in the oligosaccharide test group. These estimates are based on the preliminary data obtained under an animal research protocol at the Shriver Center, active from 1985-2000.

Typical experimental design for a suckling mouse assay

Groups	# mice/expmt.
Saline	20
Total oligosaccharides	20
Test group 1	20
Test group 2	20
Test group 3	20
Test group 4	20
Test group 5	20
Test group 6	20
Total	160

Twelve groups of oligosaccharides will be tested per year for an annual total of 1920 mice. Test groups 1-6 represent six different isolated human milk oligosaccharides or oligosaccharide fractions to be tested for biological activity, i.e., their ability to inhibit stable toxin-induced diarrhea. Replicates of these experiments are needed to confirm the efficacy of active components as well as to test additional isolated milk oligosaccharides. This study is repeated in years 2-5 to test additional oligosaccharides as they are synthesized and to confirm and strengthen the statistical confidence of positive results.

2) Although the studies on the mechanism of action of stable toxin of enterotoxigenic *E. coli* (ST) are performed in vitro, the ability of our test oligosaccharides to inhibit diarrhea, where the interactions among many complex cell types are involved, must be studied in vivo. The only known animal model is the 2- to 4-day-old suckling mouse. Only two suckling mouse animal models are accepted for use in investigating the effects of ST. One is the method of Giannella (89). ST is injected percutaneously into the stomachs of suckling mice. The mice are euthanized after a fixed period of time, typically 2-8 hours, the animals are eviscerated and the vicera and carcasses of groups of animals (typically, 4-6) are weighed. The ratio of the viscera to the carcass weights is the dependent variable. The second model, described by Cleary et al. (44), also utilizes suckling mice. ST is administered orogastrically and the animals are observed for morbidity and mortality for an 8-hour period, whereupon all animals are euthanized. Mortality over an 8-hour period is the dependent variable. We have compared these two techniques and found that the latter has the advantage of allowing fewer animals to be tested as the outcome of each individual is an independent data point, as opposed to the former where 4-6 animals are needed for each data point.
This assay has been used in all of our diarrhea-related research on the ST protective factor in human milk. We have modified the assay so that 20 animals per group now replace 100 animals per group by using in-house inbred C57 mice and with a humidity and temperature controlled environmental chamber.

3) Care of animals at the Shriver Center is provided by facility supervisor Tim Morrison under the direct supervision of veterinarian Margaret Delano, DVM, whose academic appointment is at the University of Massachusetts Medical School.

4) ST produces diarrhea and death in the mice; after 8 hours, all animals are euthanized. The diarrhea-associated death due to ST toxin is the dependent variable in this assay. The use of medication in this context would defeat the purpose of the assay. No agents other than the test compounds may be given to the animals, as the procedure is an assay for the ability of various compounds to protect the animals against the diarrhea induced by ST. Diarrhea-induced death is the endpoint. However, when pups are separated from their dams, they are kept in cages that are placed in a temperature- and humidity-controlled environmental unit designed to replicate conditions in their dams' nests. This provides comfort and minimizes any chance of hypothermia and/or dehydration.

5) Consistent with recommendations of the Panel on Euthanasia of the American Veterinary Medical Association, any of these neonatal mice that survive the eight-hour observation period will be anesthetized with hypothermia and euthanized by decapitation.

RATS

1) Each intestinal loop experiment will utilize 20 male Wistar rats (230±30 g). They will be kept under standardized environmental conditions (23°C, 50-55% humidity, artificial lighting between 09:00 and 21:00 h for one week prior to the experiment. They will be deprived of food but not water for 24 h before the experiment. They will be anesthetized with an intramuscular injection of ketamine/xylazine mixture (80 mg/Kg

ketamine, 4 mg/Kg xylazine). All the rats will be placed on an electrically heated pad under an overhead lamp and their rectal temperature kept at 36-38°C during the experiment. The abdomen will be opened and the first ligation made 5 cm distal to the flexura duodeni jejunalis and the second one 25 cm distal to the first. The lumen will be washed with 25 mL of pre-warmed physiologic saline to flush out luminal debris., and excess luminal fluid and excess residual fluid removed by gentle manipulation. The loop will be retuned to the abdominal cavity. After 30 minutes the experiment will be started by filling the loop with 2 mL of the test solution. The loop will be replaced in the abdominal cavity and the abdomen closed. The animals will be observed during the absorptive period of 30 minutes to ensure that the anesthetic does not wear off. The animals will be euthanized using carbon dioxide gas.

2) The studies proposed will determine the effectiveness of oligosaccharides isolated from human milk as inhibitors of diarrhea due to E coli heat stable toxin (ST), and to see if they play a role in maintenance of fluid balance in the intestine by their effect on guanylin, of which ST is a long-lived superagonist. To do this, it is necessary to obtain quantitative determinations of fluid secretion by the small intestine, the increase due to ST or guanylin, and the reversal of this increase in the presence of milk oligosaccharide. Such studies can only be performed in vivo, by measuring fluid accumulation in ligated intestinal loops. Rats have been used effectively to measure intestinal fluid secretion due to ST and guanylin in the published literature. Suckling mice are known to be sensitive to ST, but they are too small and fragile for use in the construction of intestinal loops. Other species that have been used for this purpose are rabbits and piglets; they are larger, more expensive to buy and house, and are unlikely to provide information that cannot be obtained by using rats.

It is possible to obtain qualitative information about which sugars are effective inhibitors of the pathways involved, and about their effect on secreted electrolytes in cell culture, and these studies will be performed before the animal studies. We will use only the most effective inhibitors in animals and use the minimum number of animals needed to provide statistically significant information. Computer simulation and non-living models cannot be used for this type of study. The procedure involves intestinal surgery, the construction of ligated intestinal loops, and the removal of the intestine for measurement of accumulated fluid, therefore it will be terminal in all cases.

Typical experimental design for a rat intestinal loop experiment

Groups	# rats/group
Buffer	5
ST or guanylin	5
Oligosaccharides	5
Oligosaccharide+ST or guanylin	5
Total per experiment	20

Either ST or guanylin will be used in a single experiment. Assuming that 5 oligosaccharides or combinations will be tested at three different concentrations, this will result in a total of 30 experiments with a total of 600 animals. Experiments yielding statistically significant data will have to be repeated, for a total of 5 more experiments and 100 animals. Therefore, a total of 700 animals will be used over the course of the grant.

Data, calculated as mean±SEM, will be subjected to statistical analysis by analysis of variance (ANOVA) with post hoc test with unpaired t-test. $P<0.05$ is considered significant.

3) Care of animals at the Shriver Center is provided by facility supervisor Tim Morrison under the direct supervision of veterinarian Margaret Delano, DVM, whose academic appointment is at the University of Massachusetts Medical School.

4) Animals will be anesthetized with an intramuscular injection of ketamine/xylazine mixture (80 mg/Kg ketamine, 4 mg/Kg xylazine) and will be observed to ensure that they are fully anesthetized prior to initiation of the procedure and during the course of the procedure. A further injection will be given immediately if the animal shows any signs of a pain reaction.

Viral Gastro nteritis Project

A. Specific Aims

The hypothesis of the project is that characterization of antibody and non-antibody factors in human milk against calicivirus and rotavirus will facilitate development of strategies to control and prevent gastrointestinal infection and illness in children. This hypothesis will be addressed by the following specific aims.

1. Characterize the phenotypic expression of histo-blood group antigens in children that are associated with risk of calicivirus infection and relate this association to histo-blood group genotypes.
2. Characterize factors in human milk that block calicivirus binding to histo-blood group antigens and examine the association between concentration of such factors in human milk and prevention of childhood infection with caliciviruses.
3. Isolate native lactadherin from human milk and determine the mechanisms by which this molecule protects infants from rotavirus infection.
4. Perform in vitro and in vivo experiments to test naturally occurring factors as well as synthetic compounds in preventing calicivirus and rotavirus binding to cell surface receptor(s).
5. Determine relative contributions of antibody vs. non-antibody factors in human milk in protection of infants from calicivirus and rotavirus infections.

B. Background and Significance

Importance of viral gastroenteritis in child health. Viral gastroenteritis is one of the most common causes of morbidity and mortality in children. On a worldwide basis, between 500 million and 1 billion episodes of gastroenteritis of all causes occur each year in children under 5 years of age, of which 130 million episodes (13 to 25%) are caused by rotavirus (RV) (1). Since the cloning of Norwalk virus (2) and subsequent development of molecular diagnostic assays, caliciviruses (CVs) have been recognized as the second most important cause of viral gastroenteritis in children (RV remains the first). Seroprevalence studies show that children are infected at an early age (3-5). The infection rates increase steadily before and during school years, reaching almost 100% by adulthood (3-9). Detection of viral RNA by RT-PCR showed that CVs cause up to 22% of sporadic cases and 5-11% of hospitalized cases of acute gastroenteritis in children (10-13). Our recent studies showed that children can be infected by CVs up to five times in the first two years of life (Progress Report). In summary, best estimates suggest that between 20% and 50% of the worldwide morbidity from diarrhea, depending upon severity, can be attributed to RV and CV.

Research needs for CVs. Norwalk virus (NV), the prototype CV, was discovered in the early 1970s (14-16), but due to a lack of cell culture and animal models to study the virus, many aspects of CVs, such as host-specificity, immunology, pathogenesis, and virus replication remain unknown. The cloning and sequencing of the prototype NV and subsequent development of molecular diagnostic assays for CVs have allowed new approaches to be used to study these issues.

Genetic and antigenic classification of human CVs. Over the past ten years, our understanding of the genetic and antigenic variation of human CVs has been greatly expanded. Several hundred distinct human CV sequences have been published or deposited into the GenBank with the number continuing to increase. According to the International Committee on Taxonomy of Viruses (ICTV), human CVs belong to two of the four genera of *Caliciviridae* (17-19). The two human CV genera are "Norwalk-like viruses" (NLVs) and "Sapporo-like viurses" (SLVs), which are further divided into genogroups and genetic clusters. Known genogroups of human CVs include Norwalk virus genogroup and Snow Mountain virus genogroup, or genogroups I and II (20, 21). From 10 to 20 genetic clusters have been reported in each genogroup or genus (Progress Report) (20, 22, 23) with new strains of human CVs continuing to be reported. The antigenic relationships among different genetic clusters, genogroups and genera remain to be determined.

New generation of diagnostic assays for human CVs. Since the cloning of NV in 1990, our group and several other groups have been dedicated to the developing of new diagnostic assays for human CVs. Although human CVs still cannot be cultivated in vitro, we now have assays for detection of both viral RNA and antigens. For detection of viral RNA, we designed a primer pair that detects both NLVs and SLVs by RT-PCR (24, 25). We also developed EIAs based on recombinant capsid antigens of human CVs (25-32). We now have accumulated recombinant capsid proteins for over 10 strains of human CVs representing different genetic clusters (26, 28, 30, 32-34). In addition, we recently developed a broadly reactive EIA for detection of viral antigens using hyperimmune antisera derived from animals cross-immunized with multiple strains of NLVs (35). This assay is particularly useful for large-scale surveillance of human CVs in different populations. Furthermore, we have generated monoclonal antibodies against several prototype strains of NLVs (36). These monoclonal antibodies are important for detection of type-specific antibodies against NLVs.

A breakthrough in understanding the host-specificity of CVs. NV infection has been hypothesized to be associated with a genetic factor since the early 1970s when pioneer scientists at the NIH had several unique observations from human volunteer studies and outbreak surveillance (37, 38), but the hypothesis was not proved due to a lack of experimental techniques. The successful expression of recombinant CV capsid protein for the first time allowed to study pathogen/host cell interaction by using these capsid proteins as a probe (2, 39-41). The first evidence of association of a genetic factor with CVs was obtained in our study on an animal CV, the rabbit hemorrhagic disease virus (RHDV). We observed that the RHDV specifically binds to H type 2 antigens of rabbit epithelial cells (42). We then performed a series of experiments on human CVs and demonstrated that human CVs recognize human histo-blood group antigens as receptors (43-45) (Progress Report). Different human CVs recognize receptors from distinct populations defined by ABO, Lewis and secretor types (43). Based on the biosynthetic pathways of human histo-blood group antigens, we were able to predict the target molecules (receptor) for individual strains studied (Progress Report). Recently, Hutson and colleagues also showed that NV infection is associated with human histo-blood group type in a volunteer challenge study (46).

Human milk factors block CV binding to saliva samples. Histo-blood group antigens are glycoconjugates (complex carbohydrates linked to glycolipids, glycoproteins, or other macromolecules) present on the external surface of red blood cells and mucosal epithelial cells, or as free antigens in biological fluids such as blood, saliva, intestinal content, and milk. Following the discovery of CV receptors, we extended the study to search for factor(s) in human milk that may serve as homologs of histo-blood group antigens that block CV attachment to intestinal epithelial cells. By using EIA and blocking EIA developed for saliva, we were able to detect factors in human milk that are similar to histo-blood group antigens in saliva (47) (Progress Report). This study suggested that histo-blood group antigens similar to those expressed in saliva also are expressed in human milk, and these antigens may serve as decoy receptors to prevent CVs from attaching to intestinal epithelial cells in infants who are breast fed.

Human milk oligosaccharides containing histo-blood group epitopes protect infants from CV infection. Glycoconjugates are one of the major components of human milk. The expression of milk fucosyloligosaccharides varies significantly (48), reflecting variation in the relative activities of the α1,2, α1,3 and α1,4 fucosyltransferases that are responsible for synthesis of the major histo-blood group antigens, such as the Lewis and ABH antigens. To determine if these milk oligosaccharide analogs of histo-blood group antigens are associated with protection of infants from CV infection, we examined the expression of these analogs in human milk oligosaccharides using biochemical methods. Among 93 infant/mother pairs studied, infants had significantly lower relative risk of moderate-to-severe diarrhea if the total quantity of maternal milk fucosyloligosaccharide was relatively high in specific α1,2-linked fucosyloligosaccharides (49). We also found that a relatively high quantity of Lewis^b antigen in milk was associated with protection against diarrhea due to CVs. These results are consistent with the finding that histo-blood group antigens are involved in CV infection.

Research needs for RV. Research on RV has been more extensive than research on CV. However, the withdrawal in year 2000 of the first licensed RV vaccine (live-attenuated RRV-tetravalent vaccine) from the market due to a temporal association with intussuseption (a bowel obstruction in which one segment of bowel becomes telescoped within another segment) emphasizes the need to better understand the mechanisms underlying rotavirus pathogenesis and immunology, including details of the molecular interactions between rotavirus and cells of the gastrointestinal tract and host-immunity in protection from infection.

Antibody correlate of protection. Conflicting results have been reported in the literature on the role of humoral immunity in protection against RV infection (50-53). Studies based on our longitudinal birth cohort of children consistently showed that antibody protects children from infection (54, 55). In our first study we showed that children were protected following natural infections (54). We then demonstrated that children with a serum IgA titer >1:800 had a lower risk of rotavirus infection and diarrhea and were protected completely against moderate-to-severe diarrhea (55). Children with a serum IgG titer >1:6400 were protected against rotavirus infection but not against rotavirus diarrhea. Protective antibody titers were achieved after 2 consecutive symptomatic or asymptomatic rotavirus infections (55).

One important reason for the difference in results could be in the study design. In our prospective study we had intensive monitoring of the children in the Mexico birth cohort. In the last funding period, we initiated a new cohort in the same periurban area of the Mexico City, with the same design, but with more infant/mother pairs enrolled (>300) and more frequent (every three months) collection of serum specimens from the children. We believe this new cohort will allow us to address additional questions, such as the role of homotypic vs. heterotypic immune responses to protect infants from symptomatic RV infection. The addition of the new cohort also will allow more powerful analysis of data by an increased total number of RV symptomatic infections in the cohorts during the past 15 years. Finally, the inclusion of the new cohort provides an opportunity to study dynamics of genetic and antigenic variations of the major circulating strains of RV and associated antibody prevalence in the populations.

Human milk protection against RV gastroenteritis. The association between breast-feeding and protection against RV infection due to immunologic factors in the maternal milk has been consistently demonstrated (56-59). However, the association due to a non-antibody factor remains unclear. Our previous epidemiologic studies as well as experimental data showed that lactadherin, one of the 3 major components of the human milk fat globule membrane, is associated with protection of infants from RV infection (60-62). RV has been reported to recognize sialic acid (SA) residues on the cell surface as a receptor for attachment and penetration (63-65). RV binding to lactadherin is substantially reduced by chemical hydrolysis of SA (60, 66). Presumbably, binding site for lactadherin is a complex sugar that includes SA. Therefore, lactadherin may inhibit RV infection by competitive binding as a soluble surrogate receptor for RV.

Recent advances in research on RV receptors and host-specificity (65, 67-73) suggest that additional mechanisms of lactadherin blocking of RV may exist. Analysis of a larger number of RV strains for their dependence on SA for infectivity showed that most human RVs, including some animal strains, are SA-independent (67). Some of the SA-independent strains recognize integrins on the cell surface for attachment and/or penetration (67, 68, 70, 72, 73). Integrins are complex glycoconjugates containing duplex peptides that are involved in cell to cell and cell to environmental matrix interactions and signal transduction. Integrins also act as receptors for several viral pathogens including foot and mouth disease virus (FMDV) (74, 75), coxsackievirus (76), echovirus (77), and some strains of adenovirus (78).

One common mechanism of cell adhesion and ligand-receptor interaction by integrins is the tripeptide RGD mediated molecular interaction. Human lactadherin contains the RGD motif in the EGF-like domain at the N-terminus of the protein and the motif is presented in a stable loop on the molecule (79-82), which is believed to have the similar function as that of cellular integrins. In addition, human lactadherin contains another two domains that share sequence homologies with factors V and VIII in the C1/C2 domains (82). The C1/C2-like

domains in lactadherin were found to be responsible for binding to cell membrane phosphatidylserine (82). Therefore, lactadherin could be a multi functional cellular adhesion molecule that blocks different RVs by different mechanisms.

In our recent analysis of CV capsid sequences, we also observed a conserved RGD or RGD-like motif in the P-2 region of the capsid of all NLVs (not of SLVs) that is believed to be responsible for binding to histo-blood group antigens (unpublished). We are in the process of generating knockout recombinant NLV capsids by site-directed mutagenisis to test this hypothesis. Preliminary results showed that mutant recombinant capsid antigen containing a sequence of "SAA" in the "RGD" region of the capsid protein of strain 387 completely lost binding activity compared with its wild-type counterpart. This study is important because, if RGD-dependent binding requirement for infection is a common phenomenon for enteric viruses, lactadherin from human milk could be broadly protective against many forms of viral gastroenteritis.

Significance of current understanding. The rapid progress of the molecular virology of CVs and the development of a new generation of diagnostic assays for CVs provide important tools for studying epidemiology and immunology of CVs that is proposed in this grant. The discovery of CV receptors and blocking factors in human milk provides new approaches to studying CV/host interaction, which may result in development of new strategies to control and prevent CV associated gastroenteritis.

Recent advances in the host cell interaction and viral receptors for RV and the characterization of the lactadherin gene for potential multi functional domains of the protein provides new approaches to studying RV pathogenesis and host-immunity. The antibody responses against RV infection described in our longitudinal birth cohort studies provide a solid basis for further studies to better understand the homologous vs heterologous protection by maternal antibodies as well as antibodies acquired from natural infection.

C. Progress Report

We made significant progress in all five specific aims proposed in our last renewal of the grant.

Data relevant to Specific Aim 1: To measure antibody levels in the cohort and associate those antibodies with protection against rotavirus and calicivirus infection and illness.

ROTAVIRUS

In our previous studies of a cohort of 200 Mexican infants, we demonstrated that natural rotavirus infection confers protection against subsequent infection. This protection increases with each new infection and reduces the severity of diarrhea. The protection is likely to be RV G type-specific (54).

In this funding period, to determine whether the protection against RV infection and illness is associated with naturally acquired serum IgA and IgG antibodies, we assessed the same 200 Mexican infants for their serum antibody responses and the clinical outcome of infection and illness (55). Serum samples collected during the first week after birth and every 4 months were tested for anti-rotavirus IgA and IgG. Children with an IgA titer >1:800 had a lower risk of rotavirus infection (adjusted relative risk (aRR), 0.21; p< .001) and diarrhea (aRR, 0. 16; p=.01) and were protected completely against moderate-to-severe diarrhea (Figure 1, next page). However, children with an IgG titer >1:6400 were protected against rotavirus infection (aRR, 0.51; p< .001) but not against rotavirus diarrhea. Protective antibody titers were achieved after 2 consecutive symptomatic or asymptomatic rotavirus infections (Figure 2, next page). These findings indicate that serum anti-rotavirus antibody, especially IgA, was a marker of protection against rotavirus infection and moderate-to-severe diarrhea.

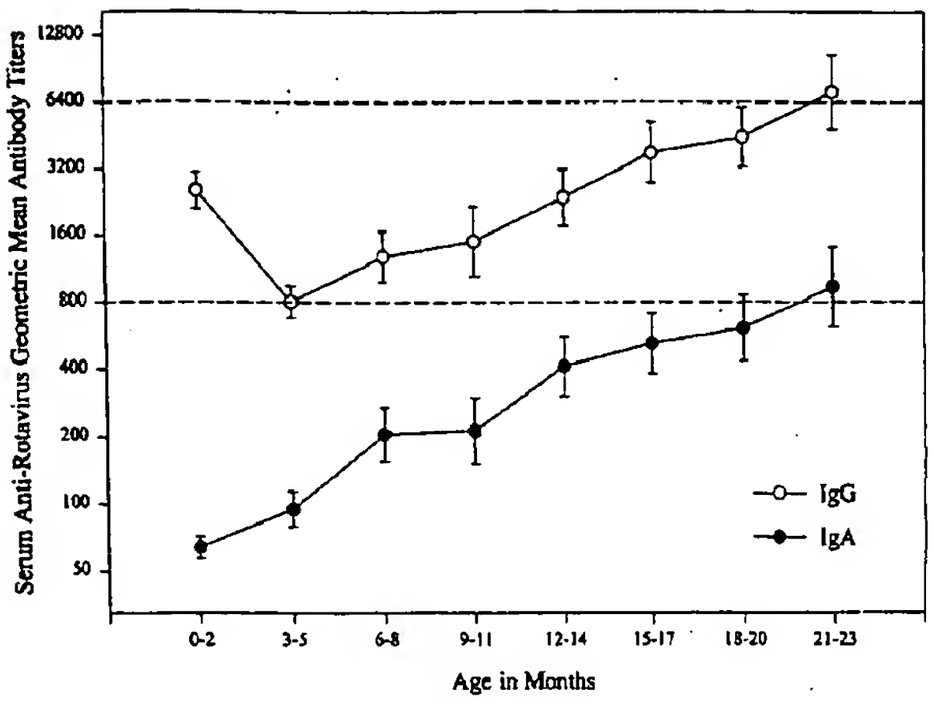


Figure 1. *Naturally acquired serum anti-RV IgG and IgA antibody titers according to age. Decrease in mean IgG titers from 0-2 to 3-5 months of age was significant (P <.001). Dashed lines indicate titers at which significant protection was achieved (1:800 for IgA and 1:6400 for IgG)*

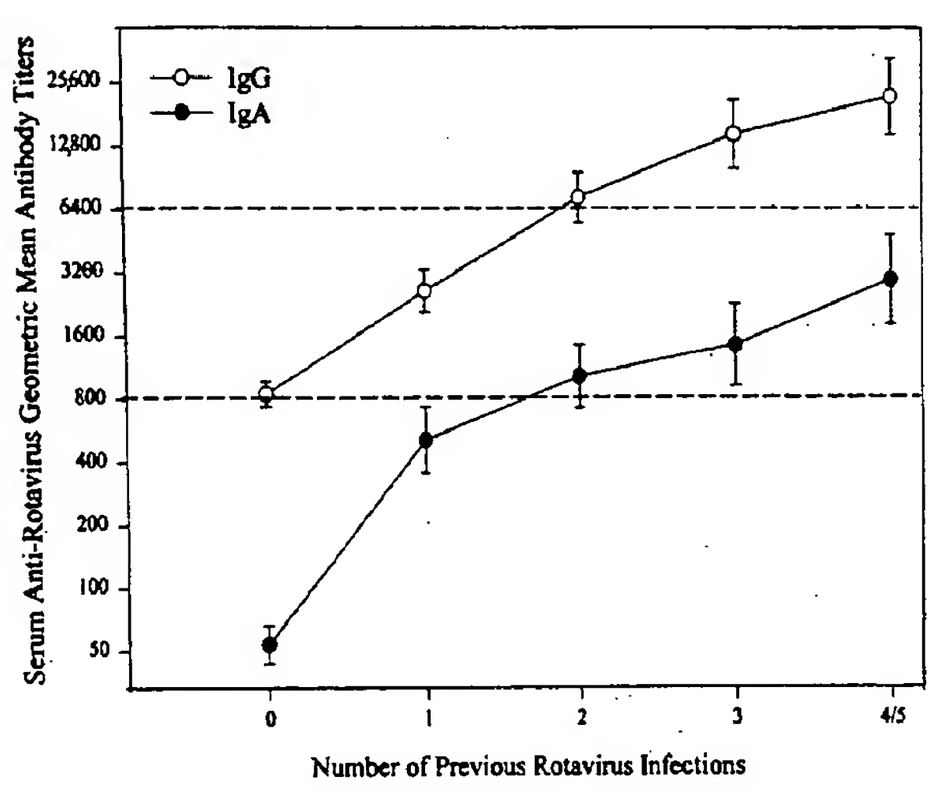


Figure 2. *Serum anti-RV IgG and IgA antibody titers, according to number of previous RV infections. Values represent geographic means, with 95% confidence intervals. Dashed lines indicate titers at which significant protection was achieved (1:800 for IgA and 1:6400 for IgG).*

CALICIVIRUS

We initiated with studies on antibody responses to CVs among patients involved in outbreaks of acute gastroenteritis using the direct antigen coating EIAs. The results showed that these EIAs detected shared antigenic epitopes among CV strains (Table 1, next page), which is not useful for typing. CVs are genetically and antigenically diverse and multiple strains of different CV genetic clusters and antigenic types co-circulate. Therefore, in the last funding period, we mainly focused on development of diagnostic assays for CVs.

Baculovirus expression of the viral capsid proteins of new strains of CVs. We expressed the viral capsid gene of 11 strains of CVs (VA98207, VA98387, VA98115, VA99452, MOH, C59, MX340, Houston4744, Hou/86, Hou/90, and Lon/92) which may belong to new antigenic types of CVs. Seven strains (VA98207, VA98387, VA98115, MOH, C59, Hou/86, and Hou/90) were successfully expressed. Four of the 7 proteins (VA98207, VA98387, MOH and Hou/90) self-formed VLPs, and 3 proteins did not form VLPs but were immunogenic and antigenic (28, 30, 32, 34). We also have two recombinant capsid antigens (rNV and rMxV) that were expressed before this grant was funded (26, 28) and three capsid antigens from our collaborators (rHV, rGrV and rDSV) (34, 83, 84). Therefore, we have a panel of 12 recombinant capsid antigens that potentially represent 11 antigenic types of CVs (the 387 and GrV may be the same type).

Table 1. Antibody responses of patients involved in twelve outbreaks of acute gastroenteritis in Virginia and the genetic identities (RNA polymerase region) of the strains involved in these outbreaks.

Location	No. patients	No. Seroresponses# (%) to rNV	rMxV	rHV	rGrV	Nucleotide identity (%) with NV	MxV	HV	LV
Pulaski Co.	1	1 (100)	0 (0)	0 (0)	NT	**76**	64	67	67
Lynchberg	3	2 (67)	1 (33)	2 (67)	NT	**76**	65	63	64
Subtotal	4	3 (75)	1 (25)	2 (50)					
VA Beach	1	0 (0)	1 (100)	1 (100)	NT	60	**94+**	75	77
Charlotte	13	4 (25)	13 (100)	12 (92)	NT	61	**96+**	76	78
Roanoke	5	0 (0)	5 (100)	3 (60)	NT	61	**73+**	83	85
Subtotal	19	4 (21)	19 (100)*	16 (84)					
Williamsberg	2	0 (0)	2 (100)	2 (100)	NT	63	76	85	**92+**
P. William	4	1 (25)	2 (50)	3 (75)	NT	62	71	80	**90**
Scott Co.	10	0 (0)	6 (60)	3 (30)	NT	62	75	82	**89+**
Arlington	11	0 (0)	2 (18)	1 (9)	NT	64	77	88	**92+**
Richmond	6	0 (0)	2 (33)	0 (0)	NT	63	75	83	**88+**
Henrico Co.	17	0 (0)	0 (0)	0 (0)	NT	63	76	87	**91+**
Subtotal	50	1 (2)	14 (28)	9 (18)					
Alexandria	2	0 (0)	0 (0)	0 (0)		62	65	65	66

Seroresponses were determined by titering acute and convalescent sera and showing 4 fold titer increase.
+, indicates positive in the rMxV (MxV column) or rGrV (last column) antigen EIAs.
* The proportions of rMxV in the VA Beach, Charlotte, and Roanoke outbreaks was significantly higher than rMxV responses in outbreaks caused by strains in other genetic clusters ($p \leq .005$ for the several comparisons) and to rNV in the same outbreaks ($P < .001$) but comparable to rHV in the same outbreaks ($p = .23$).
Bold numbers indicate the strains belong to corresponding genetic clusters of the columns.

Table 2. EIA titers of eight hyperimmune antisera against the nine rCV capsid antigens.

Antigen	Hyperimmune antisera against rCV antigens of Genogroup I NV	115	Genogroup II 207	MxV	387	GrV	HV	MOH
rNV	**8,192,000****	8,000	4,000	8,000	1,000	8,000	1,000	4,000
r115	64,000	**128,000**	1,000	1,000	8,000	1,000	1,000	4,000
rDSV*	32,000	64,000	1,000	1,000	4,000	NA	1,000	1,000
r207	4,000	1,000	**2,048,000**	32,000	64,000	32,000	1,000	64,000
rMxV	4,000	1,000	16,000	**512,000**	8,000	16,000	1,000	64,000
r387	4,000	1,000	16,000	32,000	**1,024,000**	**512,000**	1,000	64,000
rGrV	1,000	1,000	16,000	16,000	**1,024,000**	**512,000**	1,000	NA
rHV	1,000	1,000	64,000	8,000	4,000	2,000	**128,000**	64,000
rMOH	4,000	8,000	32,000	64,000	32,000	NA	8,000	**4,096,000**

* Antibody against this strain is unavailable.
** The bold numbers indicate titers against homologous antigens. The listed strains are genetically distinct (< 70% nucleotide identities in the capsid genes), except for strains 387 and GrV which belong to the same genetic cluster, the Lordsdale virus cluster.

Generation of polyclonal antibodies against individual recombinant CV capsid antigens. This has been a routine procedure in our laboratory each time we obtain a new recombinant capsid antigen. We usually use two species of animals to generate such antibodies (rabbits and guinea pigs), because it is required for a sandwich EIA to detect viral antigens in stool specimens. These hyperimmune antibodies are highly specific (Table 2), and therefore are useful for type-specific detection of CV antigens in stool specimens (25, 27, 29, 31, 85, 86). Low levels of cross-reactivities also were observed (Table 2). Such cross-reactivities were higher among strains within than between genogroups, suggesting that the antigenic relationships are correlated with the genetic classification of these strains.

Generation of monoclonal antibodies against CVs. In the past two years, we performed fusion experiments using recombinant capsid antigens from 6 CVs (rNV, rMxV, rHV, rGrV, r115 and r207) and succeeded in generation of monoclonal antibodies for all 6 strains (36). The first four antigens (rNV, rMxV, rHV, rGrV) were used to cross-immunize mice in an attempt to generate antibodies against shared antigenic types, and the last two were used separately. In the first fusion experiment, a total of 12 hybridoma clones secreting antibodies to CVs were identified. Ascites fluid was produced from 9 of the 12 clones and 8 had high titers of antibodies, among which two were specific to NV, one reacted with rMxV and rGrV, one reacted with rMxV and rHV, and the remaining monoclonal antibodies reacted at variable levels with these antigens (Table 3). In the second fusion experiment, we screened 663 hybridoma for CV VA97115 and identified 9 positive clones, and 289 hybridoma for CV VA97207 and identified 3 positive clones (data not shown). Experiments to further characterize these clones are in progress. In addition, we previously generated monoclonal antibodies against rNV and rMxV (87, 88). These monoclonal antibodies also will be used in the type-specific assays.

Table 3. Antibody (IgG) titers of monoclonal antibodies against four recombinant CV capsid antigens as determined by enzyme immune assays.

	Antibody titers (IgG) against			
MAb	rNV	rMxV	rHV	rGrV
6F11	128,000	<500	<500	<500
9C3	32,768,000	<500	<500	<500
4D4	<500	4,096,000	<500	8,192,000
17B9	<500	2,048,000	4,096,000	<500
16F4	8,000	<500	128,000	<500
13D9	4,000	<500	32,000	128,000
1B5	4,000	<500	4,000	128,000
9C12	<500	<500	64,000	<500
11D2	<500	<500	<500	<500

Antigenic characterization of CVs with monoclonal antibodies. The monoclonal antibodies against four CVs (rNV, rMxV, rHV and rGrV) have been used in EIAs to characterize antigenic relationships among different CVs (Table 3). We also used these monoclonal antibodies to develop epitope-blocking EIAs to measure type-specific antibodies against CVs in serum and human milk samples (36). Significant correlation of the antibody titers detected by the epitope-blocking EIA and the antigen capture method was observed (data not shown).

Preliminary studies of CV infection and immunity in children. Because of the genetic and antigenic diversity of CVs and co-development of diagnostic methods during the studies, only subsets of specimens from the cohorts have been tested. Preliminary results showed that high rates of CV infection occurred in children.

Cohort study of diarrhea in Mexican infants. In the first set of 115 diarrhea specimens tested by RT-PCR, 15 (13%) were positive for CVs, among which 9 (60%) were NLVs and 6 (40%) were SLVs (11). We then tested an additional 625 diarrhea specimens for CVs by RT-PCR with updated primer pairs and found 108 (17%) positive (89). The detection rate was improved by a newly developed type-common EIA (89). A total of 126 diarrhea specimens (20%) were positive. According to RT-PCR and EIA, in a total of 112 children studied from birth to 2 years of age, 65 (58%) had one, 27 (24%) had two, 9 (8%) had three, 10 (9%) had four, and 1 (1%) had five CV infections. These results indicate that CVs commonly infect infants and that further studies to characterize the infection and immunity are necessary.

A wide genetic diversity of CVs were found among children involved in the Mexico cohort. The RT-PCR products of the viral RNA polymerase of 59 strains detected in children involved in the previous and current

Mexico cohorts were cloned and sequenced (89). Forty-three (73%) were NLVs and 16 (27%) were SLVs. In comparison of the sequences, several important observations were made: 1) CV strains (Lordsdale, Southampton, Hawaii, Mexican) commonly circulating in adults also were found in children, 2) similar strains were found in both cohorts which occurred 10 years apart, 3) strains with unique sequences that potentially belong to new genetic clusters were found, 4) CVs were detected in 2-3% of non-diarrhea stool specimens, and 5) CV strains from non-diarrhea stool specimens did not reveal significant sequence difference from diarrhea stool specimens.

Data relevant to Specific Aim 2: To examine factors in human milk that are associated with protection against rotavirus and calicivirus infection and illness in the sucking infant.

Human milk lactadherin protection of children from RV infection. This project was initiated in the last funding period based on our observation of human lactadherin protecting infants from RV infection. The first evidence supporting this conclusion was based on experimental results that macromolecular components of human milk inhibit replication of RVs in tissue culture and prevent development of gastroenteritis in an animal model (60). The antiviral activity is associated with a 46-kD glycoprotein isolated from human milk mucin, known as lactadherin (60). This protein specifically binds to RV-infected cell monolayers and the binding was reduced substantially after chemical hydrolysis of sialic acid (60).

In our second study, we tested whether human milk lactadherin provides protection to children from RV infection by comparison of symptomatic and asymptomatic RV infections among children involved in the Mexico birth cohort (61). Among 31 breast-fed infants who developed RV infection, 15 were symptomatic and 16 were asymptomatic. The median concentration of lactadherin in the milk samples (obtained 4-41 days (median 13) before the infection) was 48.4 (range 5.6-180) μg/mL in the asymptomatic group and 29.2 (6.2-103.4) μg/mL in the symptomatic group. In logistic regression analysis adjusted for age at infection and secretory IgA concentration, there was a significant difference between the groups (p=0.01). No association was found between symptom status and concentrations of anti-RV Ig A, butyrophilin, or mucin (61). We concluded that protection against RV by human milk is associated with the glycoprotein lactadherin. This association is independent of products of the secretory immune system.

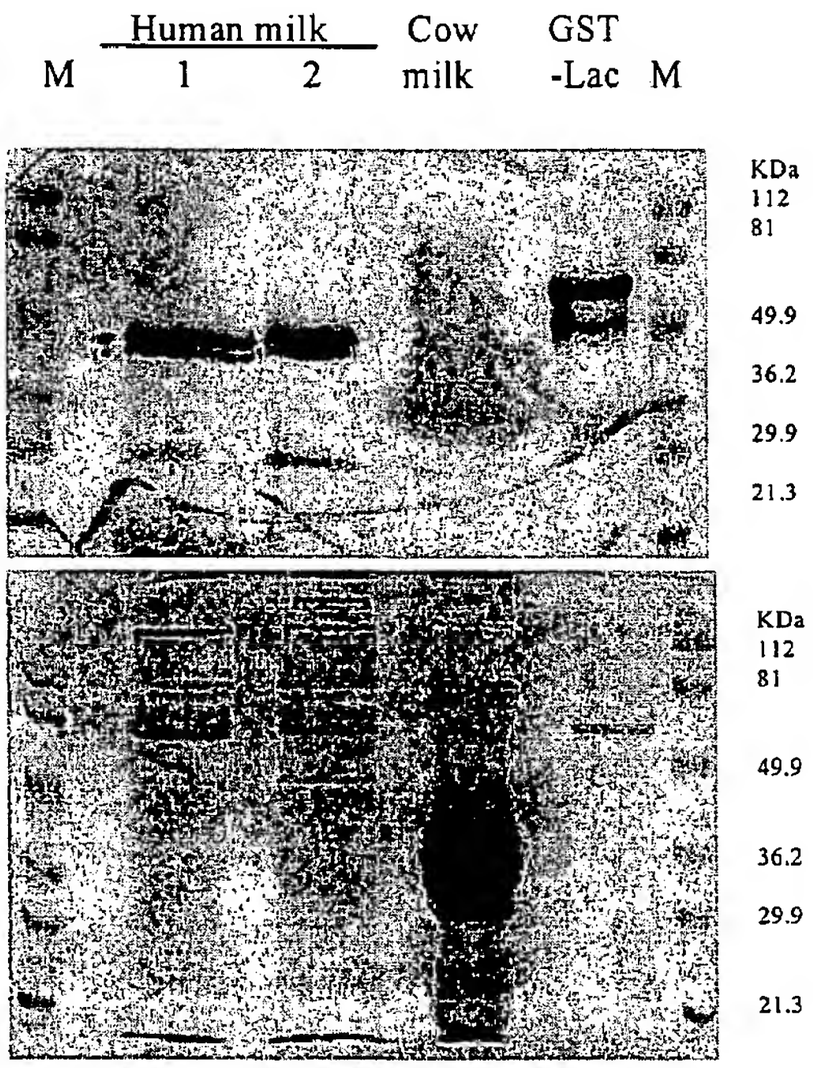


Figure 3. *Detection of lactadherin (top panel) in human milk (lanes 1 and 2) and expressed in the bacterial GST system by antibodies against the recombinant lactadherin. The bottom panel shows the same gel by Commasie blue staining. M, molecular markers; a cow milk was used as a control.*

Cloning and expression of human lactadherin gene. To further study the mechanism of this protein interaction with RV and possible other enteric pathogens, we cloned and expressed the human lactadherin gene in baculovirus and bacterial expression systems. A low yield of the protein was obtained in the baculovirus (data not shown). A high yield was obtained in the bacterial expression, but the protein was insoluble. After trials with different soluble reagents we were able to partially solublize the protein by using high concentrations of urea and quanidinium reagents. One of our purposes for expressing this protein was to generate hyperimmune antibodies for further characterization of the native lactadherin in human milk. By using the solublization methods described above as well as gel separation technique, we were able to obtain a large quantity of the protein for immunization of animals. Hyperimmune antibodies from two species (rabbits and guinea pigs) now have been

made (Figure 3) and these antibodies are being used to purify the lactadherin from human milk samples.

Characterization of virus-cell interaction and candidate receptors of CVs. This work was started in collaboration with Dr. Jacques LePendu in France. We first found that the rabbit hemorrhagic disease virus (RHDV) specifically attaches to rabbit epithelial cells of the upper gastrointestinal and respiratory tracts through the H type 2 histo-blood group oligosaccharide (42). By analogy, we tested the ability of NV to recognize such molecules on human gastro-duodenal epithelial cells and found that NV VLPs also bind to such tissue samples derived from individuals of secretor phenotype but not from non-secretor phenotype (45). Non-secretor individuals lack a functional 1,2-fucosyltransferase encoded by the *FUT 2* gene, suggesting that a fucose residue could be involved in the binding.

The specificity of NV recognizing these sugar moieties was shown by specific blocking of the binding by human milk from a secretor, by monoclonal antibodies specific for H-1 and H-3 antigens, by synthetic oligosaccharide conjugates containing secretor antigen, and by treatment of the tissues with α1,2 fucosylsidase (45) (Figure 4). NV also binds to differentiated CaCo2 cells and it is known that differentiated CaCo2 cells express human histo-blood group antigens (45). Transfection of Chinese Hamster Ovary (CHO) cells with an α1,2 -linked fucosyl-transferase cDNA allowed attachment of NV VLPs (45). The bound rNV VLPs were internalized following incubation of the cells at 37°C (45). In this study, we also developed an EIA using saliva to coat plates measuring specific binding of NV to histo-blood group antigens present in saliva (45).

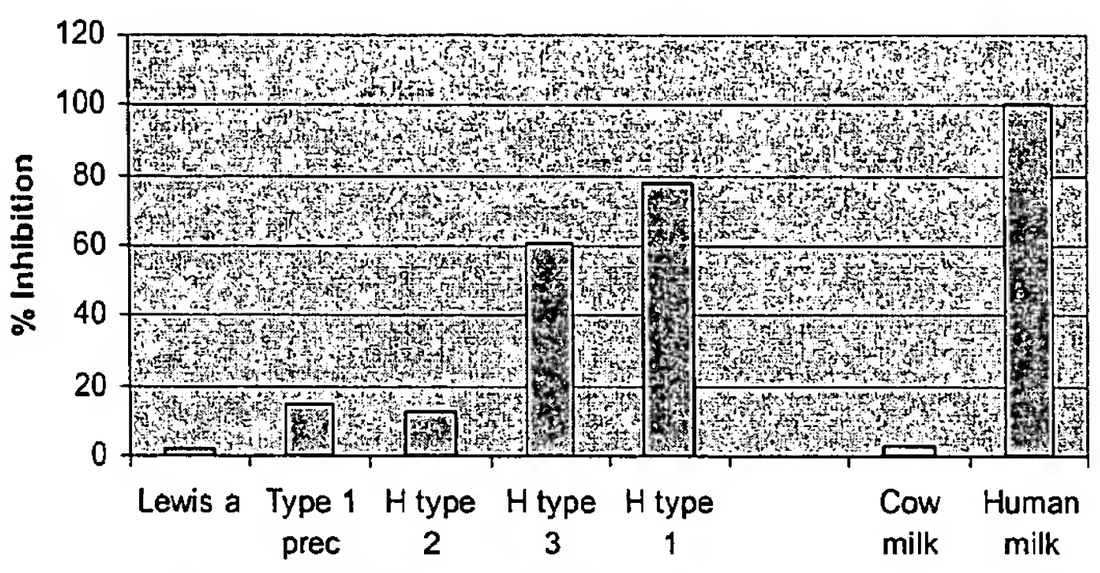


Figure 4. *Inhibition of rNV VLPs binding to saliva from a secretor individual by synthetic oligosaccharides. VLPs were preincubated with oligosaccharides at a final concentration of 1.8 mM prior to incubation on saliva coated at a dilution of 1/4000.*

We then performed a study to determine if different strains of CVs have the same host-specificity as that of NV. We tested saliva samples from 51 volunteers for binding eight recombinant capsid antigens representing seven genetic clusters of CVs with EIAs (43). The phenotypes of histo-blood group antigens of the 51 individuals were determined by EIA using monoclonal antibodies specific to Lewis^a, Lewis^b, H type 1, and types A and B blood group antigens.

To our surprise, the eight strains revealed four patterns of binding among the 51 saliva samples tested and the bindings were associated with the histo-blood group types (Figure 5). The binding activities were not associated with antibody present in the saliva because the saliva samples were boiled before being tested to inactivate antibody (43). Three patterns (strains 387, NV and MOH) bind secretors and one pattern (strain 207) reacts with both non-secretors and secretors but prefers non-secretors (Figure 5). The three secretor-binding strains can be further divided based on the ABO types: 387 recognizes all secretors (A, B and O), NV recognizes A and O, and MOH recognizes A and B.

The genetic relationships of the four strains with other CVs are shown in Figure 6 (next page). We predict that genetically closely related strains may have similar binding patterns, for example, Grimsby virus shares 98% amino acid with 387 and it had the identical binding pattern as 387 (data not shown). C59 and 115 might be similar to NV, but the recombinant capsid antigens of these two strains did not form VLP efficiently, therefore, their binding pattern remains unknown. The Mexico virus had similar pattern as MOH.

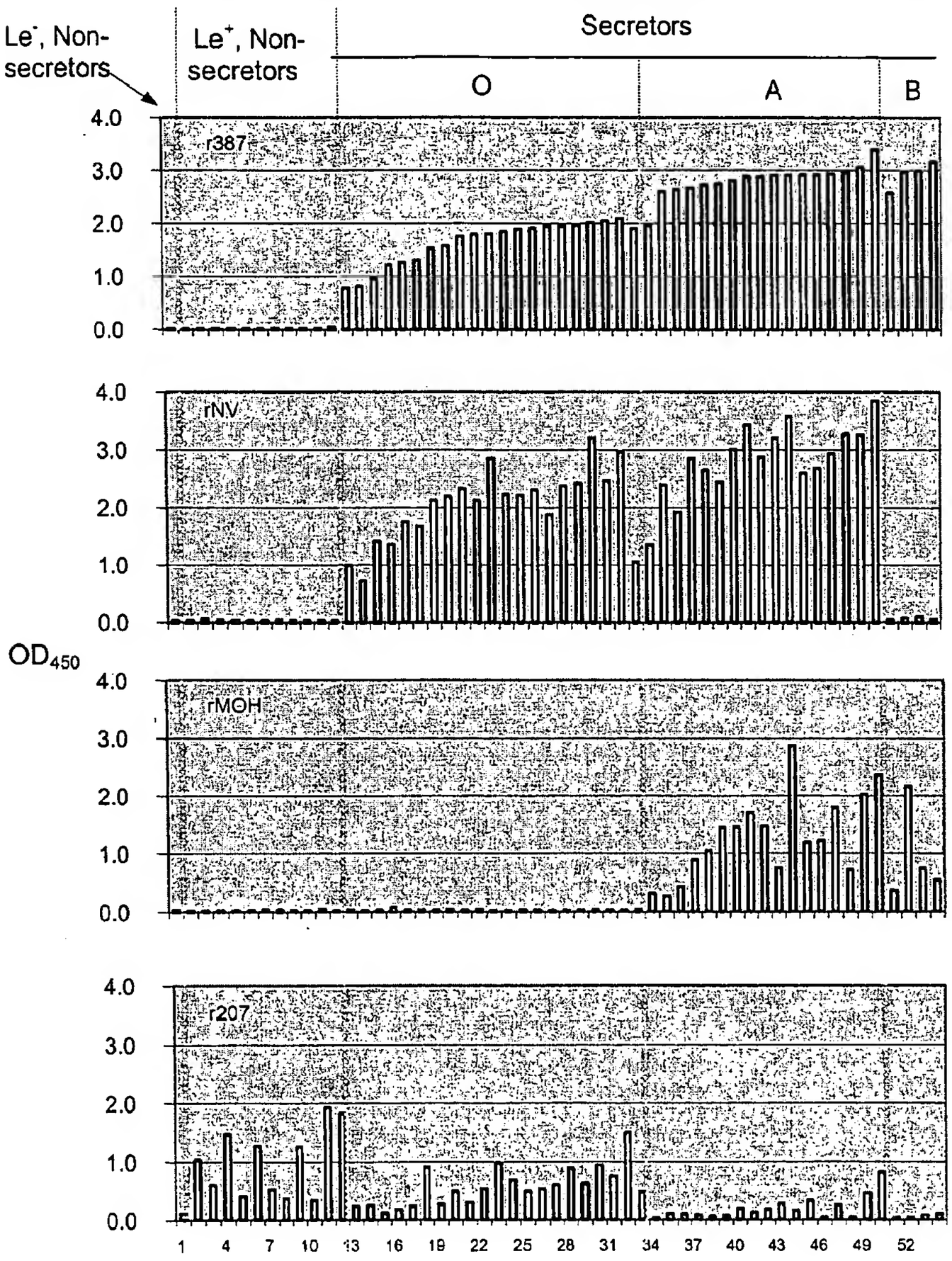


Figure 5. *Binding of four recombinant NLV capsid antigens (r387, rNV, rMOH r207) to saliva samples from 51 volunteers of European and 3 (type B) of non-European descent. The histo-blood group types of the individuals are shown at the top of the figure. Both Lewis negative (N = 6) and positive (N = 33) secretors revealed similar binding patterns and are included in one "secretor" category.*

Volunteer study with NV challenge. To investigate the association between secretor status and susceptibility to NV infection, we collaborated with Dr. Christine Moe in Emory University School of Medicine on a volunteer challenge study. Saliva samples from 50 NV-challenged volunteers were tested for secretor status and for NV-binding (90). There was a strong association between secretor status and binding ($p<0.000$) with 68% of secretors and 76% of these also binding NV capsids. Only 6% of the saliva samples from the non-secretors bound NV capsids. Secretors were almost 40 times more likely to become infected with NV than non-secretors, strongly suggesting that susceptibility to NV infection depends on secretor status.

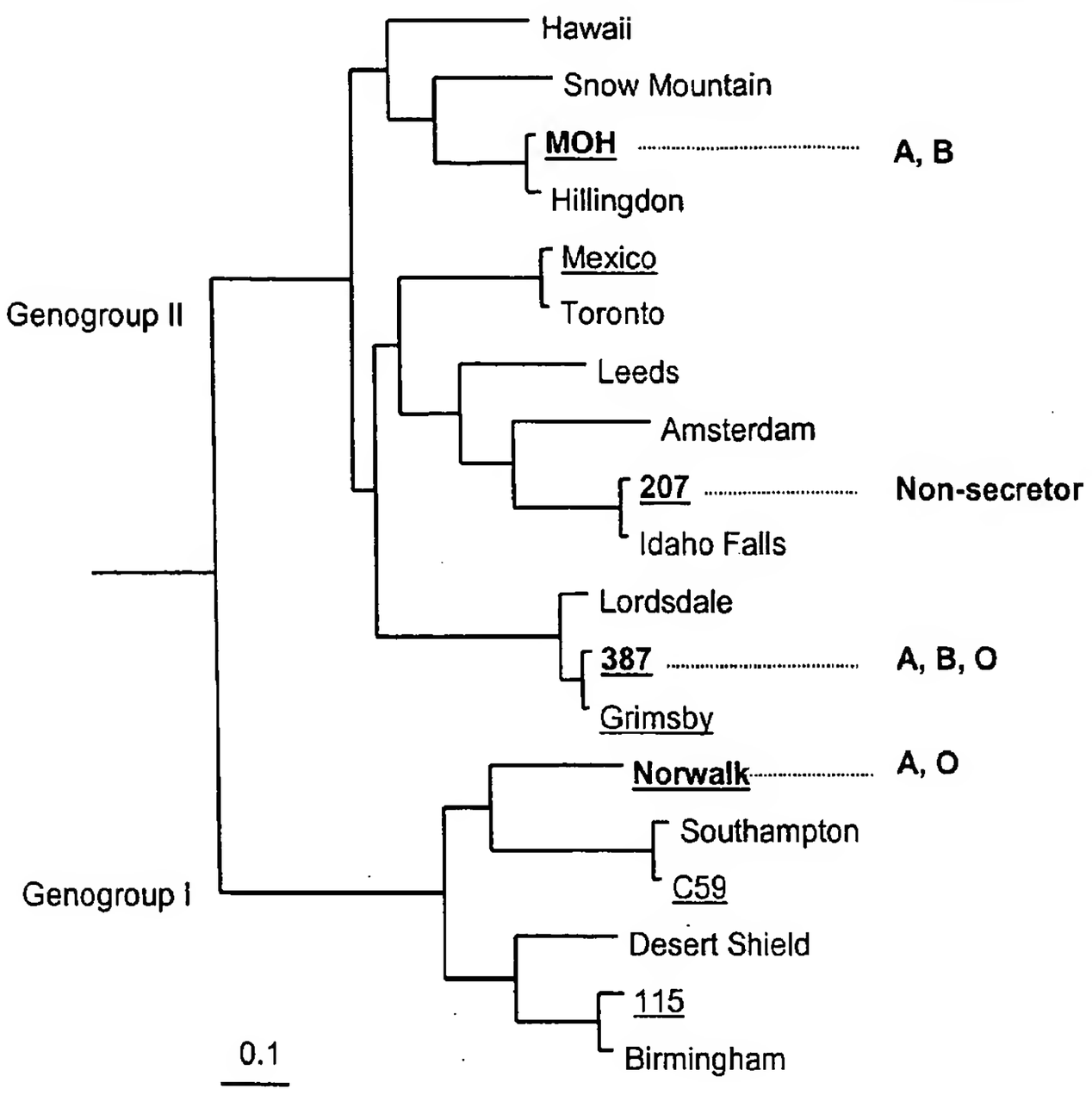


Figure 6. *Maximum likelihood phylogram (DNAML, Phylip v3.52c) of CV capsid genes. The eight strains (underlined) tested for saliva binding and their GenBank accession numbers are: Mexico (MxV, U22498), MOH (AF397156), Norwalk US (NV, M87661), C59 (AF435807), 115 (VA115, AY038598), 207 (VA207, AY038599), and 387 (VA387, AY038600). Other reference strains include Amsterdam (AF195848), Desert Shield (U04469), Grimsby (AJ004864), Hawaii (U07611), Hillingdon (AJ277607), Idaho Falls (AY054299), Leeds (AJ277608), Lordsdale (X86557), Snow Mountain (U75682), Southampton (L07418) and Toronto (U02030). The representative strains of the four binding patterns with histo-blood group types are in bold.*

Human milk contains factor(s) that block CVs binding to saliva of individuals with corresponding histo-blood group antigen types. Following the discovery of CV receptors in intestinal epithelial cells and saliva, we characterized human milk samples for their levels of activity in blocking CV binding to human saliva. A total of 60 milk samples from 30 Mexican mothers and 30 US mothers in the postpartum maternal RV vaccine trial were studied. The most dominant strain of CVs (VA387, genogroup II) was studied. Significant blocking activities (30-90% decrease in OD) were observed in 54 (90%) of the 60 mothers (47) (Figure 7, next page). The blocking was not associated with milk antibody because the milk samples were boiled before being tested to inactivate antibody. Using human milk to coat the plates, an inverse correlation of the binding and blocking were observed. The binding and blocking activities were correlated with the histo-blood group antigen types of the mothers, which were similar to those found in saliva samples (47). Cow milk did not reveal any binding and blocking activities. In conclusion, human milk contains elements that specifically block CV binding to histo-blood group antigen receptors, which may provide protection to infants from CV infection.

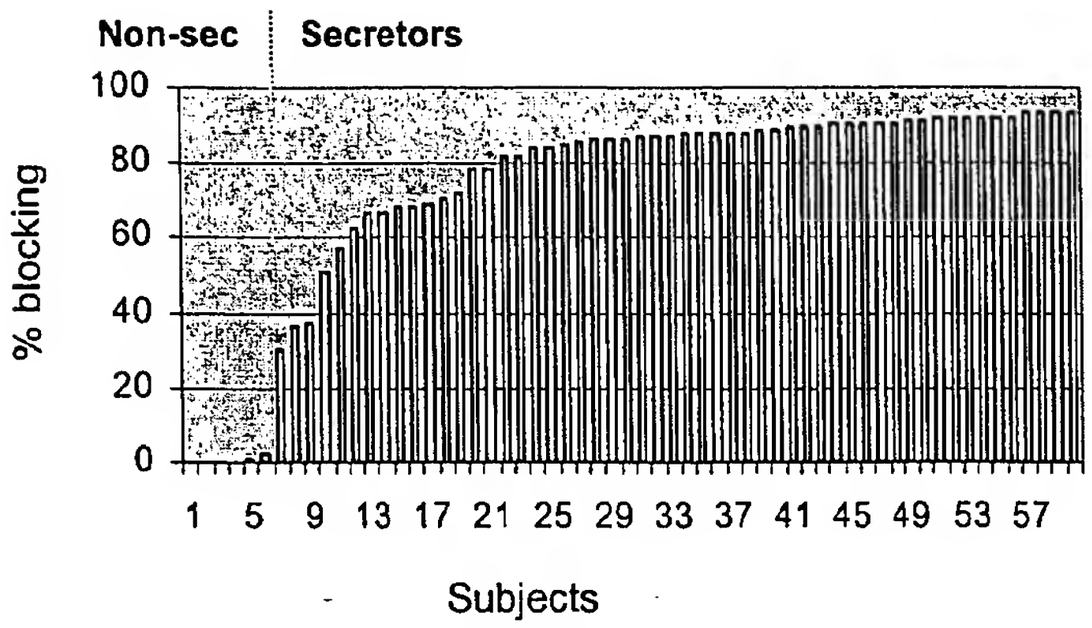


Figure 7. *Human milk blocking of strain 387 binding to histo-blood group antigens in saliva of a secretor individual. The % blocking was determined by comparing the binding OD without exposure to human milk. The blood types of the mothers were determined by EIA with monoclonal antibodies against Lewis a and Lewis b antigens.*

Human milk oligosaccharides containing histo-blood group epitopes protect infants from CV infection. To further study the role of human histo-blood group antigen association with CV infection, we characterized the α1,2-linked fucosylated oligosaccharides in human milk from 93 mothers enrolled in the first Mexico birth cohort. The results showed that infants had significantly lower relative risk of moderate-to-severe diarrhea if the total quantity of maternal milk fucosyloligosaccharide was relatively high in specific α1,2-linked fucosyloligosaccharides. Relatively high quantity of Lewis^b in milk was associated with protection against diarrhea due to CVs (n=16 cases, p=0.012). These results are consistent with the finding that histo-blood group antigens are involved in CV infection. The detailed description of the study including evaluation of other enteric pathogens is described in the Epidemiology and Glycobiology Cores.

Data relevant to Specific Aim 3: To determine whether maternal immunization with rotavirus vaccine results in enhanced protection against rotavirus diarrhea in breast-fed infants.

In our first prospective trial of maternal RV immunization, we found significant effects of vaccination of mothers with RV vaccine on milk but not on maternal serum anti-RV antibody concentrations. The significantly higher milk concentrations of antibody to RV in postpartum women who received RV immunization persisted for 4 months (91).

The above conclusion was based on a study of 33 postpartum women from Houston and two types of RV vaccines (a rhesus RV monovalent reassortant vaccine and a tetravalent vaccine). In this funding period, we repeated the study by including 30 mothers residing in Mexico City and 30 mothers residing in Norfolk, Virginia. Each individual received one dose of the tetravalent RV vaccine postpartum. A higher level and prevalence of antibodies against RV was found in the Mexican mothers than in the Norfolk mothers before immunization. Low levels of immune responses were found in both groups following immunization. These results were repeated three times for accuracy. Our final conclusion is that a single dosage of RV vaccine did not induce significant increase in antibody response in adult women. This low response may be due to pre-existing antibodies in the individuals from previous exposures to different wild type RV strains.

Data relevant to Specific Aim 4: To determine whether antibody to rotavirus non-structural protein 4 (NSP4), a potential viral enterotoxin, is a correlate of protection against rotavirus infection and illness and whether human milk contains antibody or non-antibody factors that bind to NSP4.

RV NSP4 has been recognized as the first viral toxin in the pathogenesis of RV associated acute gastroenteritis in a mouse model (92-94). To determine whether this model also applies to humans, we performed a series of studies that are summarized as follows. We used a synthetic peptide of RV NSP4 protein (amino acids 114-135) to measure antibody against this protein in children. Twenty serum specimens from children attending child care centers were tested and none were positive. We then tried to use baculovirus to express the NSP4 protein and detected antibodies in children who were infected with serotype

3 RVs using the raw culture of baculovirus recombinant infected-insect cells. However the result was inconclusive because of low levels of antibody detected. In conclusion, RV NSP4 protein might not be highly antigenic or the protein may present mainly inside cells, and not exposed to the host immune system. Thus, we moved to the following studies.

To determine whether an avirulent form of NSP4 exists, we compared sequences of NSP4 genes from the first RV infections that were asymptomatic compared to first symptomatic infections. Among 83 NSP4 genes sequenced, 35 were from RV associated with symptomatic and 48 from asymptoamatic infections. The greatest difference between NSP4s from symptomatic and asymptomatic infections occurred in the region of the previously described toxic peptide (92-94); 2 of 770 (0.3%) amino acids (aa) changed in symptomatic strains and 48 of 1056 (5%) aa changed in asymptomatic strains (compared with consensus; OR = 18, P < .001). In the same region, 1 of 34 (3%) symptomatic strains and 19 of 48 (40%) asymptomatic strains had at least one aa change from consensus (OR = 22, P < .001). In conclusion, NSP4 aa 133 differed significantly between NSP4s from symptomatic and asymptomatic first RV infections. This difference persisted when adjusted for known correlates of protection and G and P type lineage.

Data relevant to Specific Aim 5: To assess the immunomodulating effect of human milk on response to rotavirus vaccination.

This aim was eliminated due to the original budget cut in the grant. However, Drs. Ruiz-Palacios and Guerrero have conducted a randomized, controlled trial of RV vaccine in Mexican infants, and collected breastfeeding history and milk samples from approximately 300 mothers. The laboratory analyses of these specimens are in progress. This study will allow us to examine the effect of RV vaccination on maternal milk antibody response proposed in this aim.

Summary of preliminary results. In the past five years, collaboration between field study sites in Norfolk and Mexico City and laboratories in Norfolk, Cincinnati, Mexico City, Boston, Walnut City California, and Nantes, France, have generated significant new information on the viral receptors, host-specificity, milk antiviral factors, nautural history of RV infection, antibody markers for protection from RV infection, and new generation of diagnostic assays for studying CVs. With the relocation of Dr. Jiang's laboratory to the new, strong environment in the Cincinnati Children's Hospital Medical Center and the continual collaboration with different laboratories internally and externally, we are confident that the proposed specific aims will expand our knowledge of these important viral gastroenteritis pathogens and mechanisms of human milk protection.

Publications. See Core section.

D. Research Design and Methods

This section contains two parts. The first part describes the research design of the five specific aims, and the second part describes methods used to fulfill the five aims.

RESEARCH DESIGN

Specific Aim 1: Characterize the phenotypic expression of histo-blood group antigens in children that are associated with risk of calicivirus infection and relate this association to histo-blood group genotypes.

Our preliminary studies (Progress Report) demonstrated that CVs specifically bind to histo-blood group antigens present on intestinal epithelial cells and in saliva and milk. This aim will extend the study by seeking direct evidence of CV recognizing these antigens as receptors for infection. We will perform this study in children enrolled in the previous and current Mexico cohorts. The hypothesis is that, if genetic factors are involved in CV infection, the incidence of strain-specific CV infection will differ between children depending on

their histo-blood group types, i.e., secretors, non-secretors and ABO types. For the epidemiologic studies proposed in this project, we will utilize a varying number of subjects per study to maximize the efficiency of our work. Some of the studies described below use all of the subjects in both cohorts (n=622), some utilize only the breastfed in both cohorts (n=590), while the studies described below that require saliva samples and/or genetic analysis will be conducted in a subgroup of the previous and current cohort subjects who agree to participate (estimated n=415). For detailed description of these cohorts, see the Core Section, Research Design and Methods, Figure 4.

The infection status of the children will be determined by testing the diarrhea and weekly non-diarrhea stool specimens using our standard RT-PCR and newly developed more sensitive EIAs. The genetic identities of the strains will be determined by cloning and sequencing of the RT-PCR products and by type-specific EIAs. The histo-blood group antigen types of the children will be determined by EIAs on saliva samples with monoclonal antibodies.

To examine the potential for under-estimation of the total number of infections that might occur by use of stool testing only, we will test serial serum samples from a subset (n=50) of children in the cohort to identify any infections detected by 4-fold rise in serum antibody but not detected by stool sample testing. Availability of serum samples collected every 3-4 months will permit localization as to when an infection occurred. Substantial numbers (25%) of RV infections in the previous cohort were identified by seroresponse only (54, 55). If similar results are obtained in this study, the serum samples of all children will be analyzed.

Because children were monitored year-around for two years, we assume all children would have been exposed to the commonly circulating strains of CVs, including the four patterns (387, NV, MOH, and 207). We will compare risk status between the A blood group (about 20% of the Mexican population) and the O blood group (about 67% of the Mexican population). Out of our cohort of 415 participants, we anticipate 83 blood group A subjects and 278 blood group O subjects. This sample size provides >80% power to detect a 1.9 fold increased risk of CV infection in A vs O blood group children. We will also examine the risk of CV infection among genotype groups classified by their secretor/Lewis genotype combinations (see Core Aim 2 under Research Design and Methods), e.g., non-secretors, low to medium secretors, and high secretors. As described in the Core, we expect to have >80% power to detect a significant difference among these groups.

Although the monoclonal antibody-based EIAs are highly specific for blood typing, confirmation of the results with genotyping methods will be necessary because limited information is available for concordance between phenotypes and genotypes of histo-blood group antigens among Mexico populations. The genotyping methods for Lewis (*FUT3*) and secretor (*FUT2*) status of the children will be determined by saliva samples using RT-PCR followed by restriction enzyme analysis and the multiple snapshot assay. This work will be performed as part of the Molecular Biology Core.

The correlation of clinical outcome of infections (severe, intermediate and mild diarrhea and symptomatic vs. asymptomatic infections) will be assessed in relation to children's histo-blood group types. In addition, the age-specific infection rates of different strains among the children with different blood types also will be compared to determine the developmental expression of Lewis and secretor genes in newborns. Because the infection rates of the children also may be associated with the maternal antibody and content of maternal milk, the mothers' blood types and immunologic status also will be considered in the data analyses (specific aims 2 and 5). The statistical analysis will be performed as part of the Epidemiology Core.

We also propose to characterize the developmental expression of Lewis, secretor and ABO genes in newborn infants and the dynamic expression of the antigens in maternal milk. Thirty pairs of newborn infants and their mothers with different races in Mexico and Cincinnati will be followed from birth for two years. Saliva samples will be collected from the children weekly in the first month of life and then monthly until two years of age. Similar schedules of maternal milk samples will be collected. The histo-blood group antigen expression will be determined on saliva and milk samples using monoclonal antibody based EIAs. For

genotyping of histo-blood group antigens, blood samples collected at birth from the children and mothers will be tested. For the children, a second blood sample will be collected at two years of age and will be tested to confirm the genotypes. The oligosaccharide concentration in the mothers' milk samples will be studied as part of the Glycobiology Core of the grant.

Anticipated results and critique. Histo-blood group antigens have been found to be candidate receptors for several bacterial pathogens (96-107). This aim will provide direct evidence on genetic polymorphorism in the susceptibility or resistance to CV infection. In our preliminary studies, approximately 20% of tested diarrhea episodes are associated with CVs. Thus, we anticipate detection of approximately 130 CV-associated diarrhea episodes in the current Mexican cohort. In addition, we expect that testing of all asymptomatic stool samples collected weekly from each child will result in detection of 460 asymptomatic infections. Among the 590 combined symptomatic and asymptomatic infections, over 10 genetic clusters of CVs that cover all the four major binding patterns may be found, although the non-secretor binding strains may be low due to the low rate of non-secretors in the Mexico population.

Specific Aim 2: Characterize factors in human milk that block calicivirus binding to histo-blood group antigens and examine the association between concentration of such factors in human milk and prevention of childhood infection with caliciviruses.

Our preliminary results showed that human milk blocks CV binding to saliva samples. This blocking activity correlated with the blood types of the milk donors, suggesting that factors in maternal milk that are similar to those on epithelial cell surfaces and in saliva may serve as decoy receptors for CVs and therefore protect infants from CV infections.

This aim will characterize these milk factors and relate them with protection of children from CV infections. Milk samples collected from mothers involved in the Mexican cohorts will be studied. The blocking EIAs using milk samples to block CVs binding to saliva samples from individuals with known blood types described in the Progress Report will be used. The histo-blood group antigen phenotypes of the mothers also will be confirmed by testing saliva samples and by genotyping using blood samples.

In our previous studies described in the Epidemiology and the Glycobiology Cores, the concentrations of oligosaccharides, particularly the 1,2-linked and 1,3/4-linked fucosyl-oligosaccharides, varied among different individuals as well as at different lactation stages. It is important to know if these variations correlate with the Lewis and secretor antigen expression. Therefore, the dynamic of the oligosaccharide content and histo-blood group antigen expression of the mothers during the lactation stages will be characterized. This work will be performed in collaboration with the Epidemiology and Glycobiology Cores.

In our preliminary studies we observed significant variations of histo-blood group antigen activities by different methods used to prepare the milk samples for assay. To reduce such variation, a pilot study to optimize the methods for preparation of glycoconjugates, macromolecule fat globules, and free oligosaccharides will be performed. The methods described in the literature (60, 66, 79-81) and in the Glycobiology Core will be compared. After comparison, one single protocol of standard quantitative measurement of different components in human milk will be established and used in all experiments and in different laboratories involved in this program project.

We will use the combination of the current and previous cohorts (n=590) to examine milk oligosaccharide protection against CV overall as well as strain-specific protection. We will investigate the association between increased levels of specific 2-linked fucosyl oligosaccharides (H-1, H-2, Le^b and Le^y) in human milk and the decreased risk of symptomatic infection with CVs. For strain-specific protection, we will examine the three secretor-binding strains (387, NV, and MOH), which are estimated to account for >80% of all human CV infections in our study population. The 387 strain alone accounts for about 60% of all symptomatic CV infections. Assuming an incidence rate of 0.23 for symptomatic infection with 387 per child-year, the

available sample size (n=590) is estimated to provide >80% power, given ∀=0.05 and 2-tailed test, to detect a 1.6 fold relative risk comparing groups with low and high levels of 2-linked fucosyl oligosaccharide in maternal milk.

We also will investigate the protection of 2-linked fucosyl oligosaccharide in maternal milk by the joint distribution of the mother and child's blood types. We hypothesize that the infant's risk depends on the match between mother and child's blood types (concordant vs discordant pairs). For this purpose we have a potential of 415 subjects (see Core, Figure 4). Assuming that the risk for symptomatic CV infection in the concordant group is 0.2 cases/child-yr, this sample size (given ∀=0.05 and 2-tailed test) will provide >80% power to detect at least a 1.8-fold increased risk of CV infection in the discordant compared to the concordant mother-infant pairs. Data will be analyzed using chi-square or Fisher's exact test where appropriate. The relationship between protective factor and risk of CV infection will be investigated using a generalized linear model with a Poisson link function. We will also compare the mean values of protective 2-linked fucosyl oligosacchride concentrations among blood type groups.

Specific Aim 3: Isolate native lactadherin from human milk and determine the mechanisms by which this molecule protects infants from rotavirus infection.

In our preliminary studies, we demonstrated that human lactadherin inhibited RV infection in cell culture and animal model, and the expression of lactadherin in human milk associated with protection of breastfed infants from RV infection. This specific aim will determine the molecular basis of lactadherin in prevention of RV infection. We hypothesize that lactadherin may have multiple antiviral functions. The EGF-like domain of the protein may involve in the RGD- or RGD-like viral receptor bindings and the C1/C2 domain could serve as additional binding sites for the SA-dependent binding. Our long-term goals are to develop compounds that prevent binding of RV and other enteric viruses to intestinal receptors.

Testing lactadherin to block RV binding to cells by SA-dependent vs. SA-independent strains. In our early studies on the role of SA residues in lactadherin blocking RV binding, experiments were performed on RV infected-cell monolayer without separation of the viruses from the cells. Therefore, the detected binding activities could result from SA residues on the cellular proteins instead of the viral capsid proteins. According to our new hypothesis, lactadherin likely is a cell surface adhering molecule. To test this possibility, binding experiments with uninfected cells and purified viral particles will be tested separately. For the cells, different cell lines with potential different tropism for RV and from different species will be tested. For the viruses, both SA-dependent and SA-independent strains will be tested.

These experiments will be performed using native lactadherin from human milk purified by affinity columns using the hyperimmune antibodies we have generated in the last funding period. We also will perform the binding experiments with the recombinant lactadherin expressed in baculovirus and bacterial systems. If specific binding is detected, further studies to map the functional domain(s) that is responsible for the binding will be performed by subcloning and expression of the protein with deletions or point mutations.

Specific binding of lactadherin to RV and/or cellular surface proteins also will be confirmed by hydrolysis experiments. SA-dependent RV strains and/or cell lines will be studies for specific blocking of the SA-dependent infectivity by lactadherin in cell culture. If these experiments confirm our hypothesis, further studies will be conducted to determine if a special sugar moiety is involved in the binding. This study will be performed as part of the Glycobiology Core of the project. Basically, lactadherin purified from milk by affinity columns will be analyzed by mass spectrometer following removal of the protein backborne. Experiments to treat the samples with specific glycosidases (fucosylsidase, galactosidase, etc.) based on predict sugar residues also will be performed.

Determine role of RGD motif of lactadherin in blocking RV binding. The tripeptide RGD is well known being involved in cell-to-cell interaction (108-112). To determine whether the motif in the loop structure of the

EGF domain of lactadherin also is involved in blocking RV infection, blocking assays with synthetic peptides containing the RGD sequence as well as adjacent sequences of the motif will be performed. The blocking experiments will be performed using direct EIA and cell cultures with different strains of RV (SA-dependent and SA-independent strains and integrin-dependent and integrin-independent strains). In addition, peptides of the RV VP4 and VP7 motifs that are responsible for integrin binding also will be included in the studies.

Corr lation of milk lactadherin expression of mothers with clinical outcome of RV infection in the Mexico cohort children. This aim seeks direct evidence of maternal milk lactadherin in protection of infants from RV infection. The aim will be part of the studies on the relative roles of antibody vs. non-antibody factors in maternal milk in protection of children in the Mexican birth cohort study described in Specific Aim 5 of this grant (see also Core Aim 1, under Viral Gastroenteritis Project). We will use the hyperimmune antisera against lactadherin to measure the quantity of lactadherin in the maternal milk by EIA. In our preliminary study of lactadherin in human milk, we found that some women have relatively lower concentration of the 46 kDa milk protein compared with others in Western blot analysis. To replicate this phenomenon by different methods, a comparison of EIA and Western blot analysis will be performed.

Anticipated results and critique. In this study, both native and recombinant lactadherin will be characterized. The native lactadherin will be used to determine the structure and sugar composition of the molecule. Our results showed that the hyperimmune antisera raised in animals against the bacterial expressed lactadherin recognize the native lactadherin in human milk. Therefore, we are confident that we will obtain enough native lactadherin by using affinity purification procedures for the analysis. The bacterial expressed recombinant lactadherin may not be as functional as the native lactadherin for the SA-dependent binding, but may function normally for the RGD-dependent binding, because small polypeptides with variable sizes revealed blocking activities in the RGD-dependent binding assays (68).

Specific Aim 4: Perform in vitro and in vivo experiments to test naturally occurring factors as well as synthetic compounds in preventing calicivirus and rotavirus binding to cell surface receptor(s).

Specific aims 1-3 of this grant will seek direct evidences of genetic control of host-specificity and viral receptors involving in CV and RV infections. This aim will further extend our understanding of the structure/target specificity of CV and RV receptors. Our long-term goal is to develop prophylactic and/or antiviral compounds to control and prevent diseases caused by these viruses. The hypothesis is that naturally occurring or synthetic compounds that are similar to cellular receptors or mimic receptor ligands that block viruses attaching to host cells may be developed to an antiviral compound. Such compounds will be screened using currently available in vitro systems, including EIA, blocking EIA, and cell culture. Candidate reagents that reveal high binding affinity or blocking (antiviral) activities will be further tested for methods of oral administration and safety for children and adults. These experiments will be performed stepwise using animal models, human adults and then children in collaboration with the Epidemiology and Glycobiology Cores as well as the Translational Research Division of the Cincinnati Children's Hospital Medical Center.

Caliciviruses

According to the biosynthetic pathways of human histo-blood group antigens, the target antigens for each of the four patterns of CVs are known (Table 4). The antigens for the three secretor-binding strains (387, NV and MOH) have a common fucosyl residue (α1,2-linkage). Our previous experiments showed that this sugar residue plays a key role in CV-receptor recognition.

Table 4. The biosynthetic pathways of the major histo-blood group antigen types and the predicted target products by different CVs representing four major binding patterns*.

Norwalk-like viruses	Non-secretor	Secretor		
		O	A	B
	Precursor | Le^a	Precursor / | Le^a H type 1 | Le^b	Precursor / | Le^a H type 1 | A type 1 | ALe^b	Precursor / | Le^a H type 1 | B type 1 | BLe^b
387	-	+++	++++	++++
NV	-	+++	+++	-
MOH	-	-	+++	+++
207	+++	++	+/-	+/-

*For clarity, the Lewis negative types are not represented on the table. In these types, the Le^a and Le^b antigens are absent. Strain 207 does not bind to saliva from such individuals (data not shown).

We also predicted that the minimum required structure that is recognizable by CVs is the H-type 1 molecules, which contains three sugar residues (113, 114) in the antigenic epitope of the antigen. Therefore, the first compound to be tested will be oligosaccharides containing the H-type 1 with the fucosyl side chain.

In the Campylobacter subproject of this grant, synthetic α1,2-linked fucosyl-lactose (H-2) for anti-campylobacter colonization will be tested (see Campylobacter project and Epidemiology and Glycobiology Cores). This compound has the basic structure of three sugar residues including one fucose like those of the H-type 1 molecules for CVs. Therefore, we will include this structure for comparison with the CV-specific blocking compounds. After testing of the minimum required structures, oligosaccharides with additional side chains, including N-acetyl-galactosamine and galactose for the types A and B antigens, respectively, will be tested. Naturally occurring oligosaccharides or glycoconjugates in human milk prepared by HPLC fraction will be tested. We previously observed that bound oligosaccharides had a higher efficiency in blocking CVs binding than free oligosaccharides did. Oligosaccharides will be conjugated through cyanoborohydrate reduction to a human milk protein in the Glycobiology Core and their relative activity will be compared with the acitivity of the free oligosaccharides.

The saliva EIAs for measuring CV binding described in the Progress Report will be used for screening compounds for blocking activities. Among the three secretor-binding strains, 387 was the broadest one that recognizes A, B, and O secretor antigens, therefore, 387 will be used in the primary screening. Specific epitope-blocking will be determined by using individual strains for each binding pattern. The saliva binding or blocking activities determined by the blocking EIA will be further confirmed by blocking assays using cell culture with differentiated CaCo2 cells as well as fucosyltransferase gene transfected CHO cells.

To better understand the histo-blood group antigen system, we will generate transformed cell lines by transfection of human *FUT 2* gene. Functional and mutant human *FUT 2* cDNAs now have been cloned in

our laboratory (data not shown). We are in the process of testing these clones by transient transfection. Stable transformed cell lines will be established following the transient transfection experiments. Our long-term goal is to study CV pathogenesis and immunology in transgenic animal models that express human fucosyltransferases. The in vitro transfection experiments will be a necessary step in development of such animal models.

According to the biosynthetic pathways, strain 207 is the only non-secretor binder and it likely recognizes the Lewis^a antigen. However, because 207 binds to saliva of some secretors and the binding pattern was not highly correlated with that of monoclonal antibodies specific for Lewis^a antigen (our unpublished data), additional molecules in secretor individuals may be involved in 207 binding. To test this possibility, two approaches will be performed: 1) to characterize the antigen biochemically by using affinity purification of the antigen from saliva using 207 antigen as capture ligand, and 2) to synthesize oligosaccharides with the basic structure of Lewis^a antigens with additional side chains of fucosyl residues. Both approaches will be performed in collaboration with the Glycobiology Core.

When specific compounds for blocking specific viral strains are identified, experiments will be performed to determine how these compounds can be used in one mixture to block multiple pathogens. Alternatively, additional polyvalent compounds may be designed. These compounds may contain single or multiple side chains and each side chain may contain single or multiple functional domains that bind to or mimic the receptor ligands.

Rotaviruses

According to the potential multi-function hypothesis of lactadherin proposed in specific aim 3, two types of compounds will be tested for blocking RV infection. One is to block the RGD-dependent binding and the other is to block oligosaccharide (sialic acid)-based binding. For RGD-dependent binding, synthetic polypeptides will be tested mainly. Because RV VP4 and VP7 do not contain the typical RGD motif, polypeptide with the predicted DGE motifs for RV VP4 and LDV motif for VP7 in addition to the RGD motif (68) will be tested. We also will extend the study by designing additional blocking molecules based on the CV capsid sequences, because CVs also contain RGD and RGD-like motifs in the P-2 region of the out capsid loop (39, 115, 116). These potential tripeptide motifs will include RGT, RGK, RGR, RGS, KGE and KGT based on multiple alignment of known human CVs (data not shown), which are predicted to relate with target specificity or binding affinity of CV receptors. A direct comparison of the blocking activities of these molecules with both RV and CV will be performed. The structures of the compounds to block the oligosaccharide-based binding (sialic acid-dependent) for RV will be determined in specific aim 3. Both soluble and conjugated oligosaccharides will be tested.

The in vitro assays may be varied according to different mechanisms of bindings. For testing polypeptide blocking of the RGD-dependent binding, cell lines with specific integrins on the cellular membranes will be tested. For the SA-dependent binding, SA-dependent RV strains will be tested. The binding and blocking activities will be measured by direct binding assays using cell membrane extracts and purified viral antigens. Candidate blocking reagents will be further analyzed using cell cultures with multiple strains (SA-dependent and independent). Additional evaluation will be performed in animal studies using mouse models.

Anticipated results and critique. The saliva binding and blocking EIAs are simple and highly sensitive, which should allow us to screen a large number of compounds in a short period of time. The assays need small amounts of each compound, which are readily available from the Glycobiology Core. Large-scale synthesis of individual compounds also is not difficult, but is expensive since these molecules are not produced routinely, therefore, large-scale studies will be performed only for promising candidate compounds. The difficulty for CVs is that we do not have a cell culture and animal model to test the efficacy of the drugs. We will test them by natural infection in the Mexican children in the same community of our previous birth cohort. One potential approach is to evaluate them through outbreaks of acute gastroenteritis, such as those

in child care centers and military battle ships. Our group has collaborated with US Navy (Dr. Scott Thornton, Navy Environmental and Prevention Medicine Unit No. Five, San Diego, CA) for surveillance of CV-associated outbreaks in battle ship outbreaks for the past five years. Our team also has a long history of research experiences in diarrhea outbreaks in child care centers. These potential approaches will be utilized when a good candidate compound is identified. The lactadherin is well known for its cell adhesion activities. With the finding of the RGD-dependent binding of the protein, we believe the general cell adhesion molecule has a broad-spectrum of antiviral activities to different viral families. The hyperimmune antibodies generated in our laboratory against lactadherin are highly specific and we are confident in obtaining a highly pure preparation of the molecule for further analyses. The peptide blocking is a common technique for mapping the RGD motif (117-120), therefore, we believe we will generate useful information.

Specific Aim 5: Determine relative contributions of antibody vs. non-antibody factors in human milk in protection of infants from calicivirus and rotavirus infections.

The previous four specific aims mainly study the non-antibody factors in protection of infants from CV and RV infections. This aim focuses on studying antibody response or protection of children from CV and RV infections. Children involved in the Mexican birth cohorts will be studied. Non-antibody factors such as the histo-blood group antigen types of the infants (susceptibility) and the mothers (protective effect) and the concentration of lactadherin in maternal milk (protective effect), will be considered in the data analyses. Our long-term goal is to seek useful information for vaccine development and/or prophylactic agents to prevent diseases caused by CV and RV.

Calicivirus

For CV, we will focus on basic understanding of the natural history of infection and immunity, because data are lacking in longitudinally followed children. Similar questions that were asked for RV in our previous studies in the Mexican cohort will be asked for CV, including prevalence and age distribution of CV infection, distribution of major circulating genetic and antigenic types of CV, frequency and extent of re-infections, clinical outcome of infections (symptomatic vs. asymptomatic, mild, moderate or severe diarrhea). These questions will be addressed in specific aims 1 and 2.

Following detection of antibodies in the children and mothers' milk, additional questions will be asked, such as the type-specific antibody response and protection, role of natural infection in protection from re-infection, and role of maternal antibody in protection of infants from infection in the first 6 months of life. In the previous Mexican cohort, we observed that 15%-20% of diarrhea episodes were associated with CV infection. Among these episodes, ~60% were first infections and one quarter of episodes were second infections. Testing of antibodies in serum specimens collected before the first infection will determine the baseline of antibody (mainly maternal transplacental antibody) in the children. The antibody titers in the serum specimens collected after each episode of diarrhea will indicate the levels of antibody responses following natural infections.

The direct antibody detection EIAs using recombinant CV capsid antigens as the capture antigens will be used for the general screening. For type-specific antibody detection, the monoclonal antibody-based epitope blocking assays will be performed. We currently have monoclonal antibodies against four major types of CV (NV, GrV, HV, MxV). In our separate RO1 grant we will generate monoclonal antibodies against additional circulating types. A panel of type-specific antibodies against the major CV antigenic types is expected to be produced in our laboratory within the next 2-3 years, which will allow us to detect type-specific antibody responses to at least 80% of the antigenic types.

Based on use of both previous and current cohorts (n=622) we will have more than 80% power to detect a protective effect (RR = 0.7) of a first CV infection against a second CV infection of any type. We developed a statistical method to determine natural protective rates for RV based on a modified Cox proportional hazards

time to failure model (54). The same method will be employed to assess the protective effect following multiple CV infections, to determine the number of antigen exposures needed to induce protective homotypic immunity and the number and spectrum of heterotypic protection. We also will utilize a Kappa-like statistic to examine the degree of concordance/discordance between first and repeat infections (54, 121).

Finally, determination of antibody prevalence to different strains of CV will help to clarify our hypothesis of CV recognizing human histo-blood group antigens as receptors. We hypothesize that individuals who do not have specific blood type receptors will have lower antibody prevalence against specific strains than individuals who have the receptors. We predict that strain 387 will have the highest antibody prevalence among secretors, NV the second and MOH the least; while 207 will have higher antibody prevalence than the other three strains in the non-secretor individuals.

Rotaviruses

For RVs, we will focus on type-specific antibody response against RV infection. In our previous studies, we have demonstrated two consecutive natural RV infections to be protective to children from re-infection. In this aim, we will characterize the G and P types of the strains involved in each episode. The antibody titers in the serum specimens collected before and after the episodes of the children will be determined by type-specific epitope-blocking assays against the four major G types of human RV. This design will allow determination as to whether homologous or heterologous antibody responses are induced and whether such antibodies are protective against subsequent infections. In the previous Mexican cohort, we had 260 RV infections based on RV shedding, among which 144 were symptomatic infections. The distributions of the G types are 20%, 15%, 35%, and 6% for G1 to G4 respectively. We may not have the same G types in the current cohort, but the total number of RV infection is expected to be the same. Therefore, by analyzing data of both cohorts, we will have enough infections by all four major G types for statistical analysis.

Anticipated results and critique. The studies for CVs focus on the basic understanding of the natural history of CV infection, particularly on the host immunity or protective antibodies following infection. Because CVs are antigenically diverse, understanding the type-specific immune responses and the spectrum of responses against different types will provide useful information as to whether a vaccine can be developed for CVs. Since there are no animal models for CVs, these questions only can be addressed through specially designed populations using molecular epidemiology and immunology tools. The studies for RVs will further advance our knowledge on homologous vs. heterologous antibody responses in protection of children from RV infection, which are directly related to the design and development of a vaccine against RV infection. In this study, we will try to analyze the role of antibody relative to non-antibody factors in protection of children from infection. We hope the antibody protective effect will be clearer after these non-antibody factors are removed.

METHODS

All methods and assays listed are available in Dr. Jiang's laboratory except the genotyping of blood group antigen types which will be developed in the Molecular Biology Core by Dr. Tibor Farkas under consultantion of Dr. J. LePendu, with whom we have been collaborated for the past 2 years. The methods for purification and characterization of oligosaccharides in human milk specimens will be performed in the Glycobiology Core as part of our ongoing collaboration with Dr. David Newburg. Statistical analyses will be performed in the Epidemiology Core with Drs. Mekibib Altaye and Ardythe Morrow.

Calicivirus

1. RT-PCR detection, cloning and sequencing of viral RNA. The RT-PCR protocol for detection of CVs will be used to screen stool specimens and for amplification of the viral capsid gene and other regions of the

genome. The most commonly used primer pair 289/290 (11, 24) and a recently designed degenerate primer set in the same region of the genome (unpublished) will be used for screening CVs in the Mexican cohort. Additional primers will be designed based on new sequences found in the screening. The Trizol method (11) will be used to extract viral RNA from stool specimens for the RT-PCR. The viral cDNA will be cloned into the pGEM-T vector (Promega). The cloned cDNA will be sequenced by a capillary sequencer.

2. Phylogenetic analysis of CVs. The genetic identity of the strains will be determined by comparison of their sequences to those in GenBank and our local CV sequence database. Pairwise alignments will be run using the OMIGA 2.0 package (Oxford Molecular Ltd, Oxford, UK). Phylogenetic analysis of aligned sequences will be performed using the GCG programs supplied in the network by the Bio-informatics Center at the Cincinnati Children's Hospital Medical Center (CCHMC).

3. Enzyme immune assays based on reagents derived from baculovirus-expressed recombinant CV capsid antigens. The protocol of expression of the rNV and rMX viral capsid proteins in baculovirus (26-29) will be followed for generation of recombinant capsid antigens. CV capsid proteins expressed in the insect cell culture will be purified by using sucrose and CsCl gradient centrifugation. The purified viral proteins will be used to immunize guinea pigs and rabbits to produce hyperimmune antisera following the procedures described previously (26-28). The viral antigens will be used to immunize animals separately to generate type specific antibodies or mixed to generate cross-reactive antibodies against multiple antigens (122).

4. EIA to measure type-specific antibodies against CVs. Epitop-blocking EIAs using Mabs (36, 123, 124) will be developed to detect type-specific antibodies against CVs in serum and milk specimens. Mabs specific for NV, HV, MxV, 387 listed in Table 3 will be used to develop the optimal conditions for the assays. Serum specimens from patients infected with homologous strains of CVs and from patients infected with reference strains representing different genetic clusters will be tested as reference to evaluate these assays. Mabs against other types of CVs also will be generated in our separate NIH funded project (NIAID AI-37093). Similar epitope-blocking EIAs will be developed using these new Mabs. Mabs generated against new strains of CVs will be characterized using direct EIA, competition EIA, Western blot analysis, and immunoprecipitation using the procedures described previously (36).

5. Measurement of recombinant CV capsid antigen binding to saliva samples. The EIAs using baculovirus-expressed recombinant capsid proteins of CVs to detect histo-blood group antigens in saliva samples developed in our laboratory will be used. The four strains (NV, 387, MOH and 207) that represent four saliva binding patterns will be studied (43, 45). Saliva samples will be boiled and centrifuged, and the supernatant will be stored in –20°C until use. For testing rCV binding to saliva, microtiter plates (Dynex Immulon, Dynatech) will be coated with saliva samples at a dilution of 1: 1,000-5,000 in phosphate buffer saline (PBS). After blocking in 5% dried milk (Blotto), rCV capsid proteins at ~1.0 μg/mL in PBS will be added. The bound rCV capsid proteins will be detected by using a pooled guinea pig anti-CVs antiserum, followed by addition of horseradish peroxidase (HRP)-conjugated goat anti-guinea pig IgG (ICN, Aurora, OH). In each step, the plates will be incubated for 1 h at 37°C and washed five times with PBS. The enzyme signals will be detected by the TMB kit (Kirkegard & Perry Laboratories, Gaithersburg, MD) then read at a wavelength of 450 nm using an EIA spectra reader (Tecan, Durham, NC) as described by the manufacturer.

6. Determination of Lewis, secretor and ABO blood types. Saliva samples will be diluted at 1:1,000 in PBS and then coated onto microtiter plates (Dynex Immulon) overnight at 4°C. After blocking with 5% Blotto, monoclonal antibodies specific to Lewis, secretor, type A, and type B antigens will be added. After incubation for 1 h at 37°C, HRP-conjugated goat anti-mouse IgG or IgM antibodies will be added. Following each step, the plates will be washed 5 times with PBS. The color reaction will be developed and recorded as described above. Commercially available monoclonal antibodies (MAbs) specific to human histo-blood group antigen types will be used for phenotyping. MAbs BG-4 anti-H type 1, BG-5 anti-Le^{a}, and BG-6 anti-Le^{b} were purchased from Signet Pathology Systems (Dedham, MA). MAbs

BCR9031 anti-H type 2, BCR 9010 anti-A, and BCRM 11007 anti-B were purchased from Accurate Chemical & Scientific Corporation (Westbury, NY).

7. Genotyping histo-blood group antigen types by PCR. The commonly used polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method will be used for typing the common non-functional alleles that are well characterized in Caucasians and other race groups (125-132). DNA will be extracted from the blood or saliva samples by the Trizol method. The coding sequence of the *FUT 2* gene will be amplified by PCR using specific primers followed by restriction enzymes analysis, which cuts the functional but not the mutated alleles or vise versa. The sizes of the bands obtained after electrophoresis indicate whether the individual is homozygous or heterozygous for the functional allele, or homozygus for an inactivated allele. For mutations that are not detected easily by PCR-RFLP, multiplex snapshot genotyping method will be utilized (125-132).

8. Binding and internalization of NV VLPs on CaCo2, transfected CHO and other cells. The procedures described in the rNV binding study (45) will be followed. Confluent monolayers of CaCo2 and other cells will be washed and then chilled to 4°C. Increasing amounts of ^{35}S labeled VLPs will be added to the wells and the cells will be incubated at 4°C for 1 h. The binding will be terminated by washing the cells with cold medium. To determine specific binding of VLPs, separated wells will be preincubated with 20-fold excess of unlabeled VLPs before labeled VLPs. The specific binding will be calculated by subtracting the nonspecific binding from the total binding. For internalization experiment, the cells will be transferred to 37°C for one hour after the one hour binding at 4°C. The non-internalized VLPs on the cell surfaces will be removed by proteinase K treatment before solubilization of the cells to analyze the internalized VLPs.

Rotavirus

9. EIA for detection of RV in stool specimens. Commercial assays are utilized (Premier Rotaclone, Meridian Diagnostics Inc.)

10. EIA-based G typing. The monoclonal antibody-based EIA for G typing uses typing monoclonal antibodies prepared by Taniguchi, Coulson, Greenberg, and Birch as capture antibodies (133-136). This panel provides several monoclonal antibodies for each of G types 1, 2, 3, and 4. A monoclonal antibody specific for a common epitope on VP6 is included as a control to assess antigen quantity. A homologous hyperimmune guinea pig antiserum serves as detector antibody for each typing monoclonal antibody. Cut-off points are determined for each monoclonal antibody using RV-negative stool extracts and viruses of heterologous types.

11. RT-PCR typing. RT-PCR will be performed to determine P and G genotypes. Viral RNA for RT-PCR will be extracted from stool suspension using the Tryzol method. For RT-PCR, the protocol follows that of Gouvea, et al (137), using the same primers for G genotyping. The method for P genotyping is that described by Gentsch and co-workers (138), using a strategy similar to that for G genotypes.

12. Serum VP7 type-specific epitope blocking activity. VP7 type-specific antibodies will be detected by an epitope-blocking EIA (123, 139). Hyperimmune guinea pig antiserum to one of the human RV VP7 types 1,2,3 or 4 is used to capture homologous viral antigen. After an overnight incubation, serum diluted 1:10 and 1:40 is added. Control wells received either PBS-BSA-FCS only (no blocking), homologous serum from hyperimmunized guinea pigs (complete blocking), or serum from a patient with a known titer (internal standard). After a wash step, an optimal dilution of competing monoclonal antibody to the homologous G type is added. Epitope-specific monoclonal antibodies used will be selected from the panel available to us. The epitope blocking results are reported as the percentage decrease in optical density value of the test serum at a 1:10 dilution when compared to the non-blocking control.

13. Lactadherin concentration determination in milk. Lactadherin is quantified by end-point dilution (61) using a slightly modified protocol. Serial dilutions of milk samples are tested in an EIA using guinea pig anti-lactadherin antibody raised against recombinant lactadherin. Concentrations of lactadherin are estimated from a standard curve constructed from testing dilutions of purified lactadherin. Butyrophilin tesing is

performed in parallel, using a butyrophilin-specific monoclonal antibody and purified butyrophilin, for comparison.

14. Experiments to determine the structure and oligosaccharide composition of lactadherin will be performed as described in the Glycobiology Core.

SUMMARY

This renewal will continue research on the two most important viral gastroenteritis pathogens in children, the RV and CV. The research of CV has just passed its first decade following the cloning of the prototype NV in 1990. The research of RV faces new challenges after withdrawal of the first licensed live attenuated RV vaccine from the market. However, recent advances in understanding of the host-specificity and cellular receptors for both CV and RV provide new approaches to overcome these challenges. Our proposed studies will follow five lines of experimentation on related topics of natural history of infection and immunity, maternal protection, host-specificity, molecular basis of pathogen-receptor interaction, which will lead to new strategies to control and prevent CV and RV infection and illness. With the continual team-work spirit of the investigators, the support of the collaborators nationally and internationally, and the new environment of the research laboratory in Cincinnati, we are confident that significant progress toward advancing this field will result.

Time table for the proposed studies

	Year 1	Year 2	Year 3	Year 4	Year 5
Aims					
1. Determine CV infection in Mexican cohort					
Determine genetic identity of detected CV strains					
Characterize histo-blood group phenotypes of Mexican children					
Determine histo-blood group genotypes of Mexican children					
2. Characterize histo-blood group phenotype of Mexican mothers					
Measure histo-blood group epitopes in milk of Mexican mothers					
Determine histo-blood group genotype of Mexican mothers					
3. Purify lactadherin from human milk by affinity column					
Determine effect of lactadherin in blocking RV infection					
Determine concentration of milk lactadherin in Mexican mothers					
Map domains in lactadherin responsible for RV binding/infection					
4. Block CV binding to blood antigens with oligosaccharides					
Block RV binding and infection with synthetic polypeptides					
Clinical trials for safety of candidate antiviral compounds					
5. Establish type-specific assays for CV antibody detection					
Measure type-specific anti-CV antibodies in Mexican children					
Measure antibody response to RV before and after infection					
Analyze data of RV and CV infection and antibody responses					

E. Human Subjects Research

1. Risks to the Subjects

Human Subjects Involvement and Characteristics: Human subjects have been enrolled in Mexico as outlined in the research design and methods section of the Core. These subjects have provided stool and milk

specimens as well as the accompanying demographic data for the projects. In Cincinnati, mother In Mexico, mother-infant pairs will be enrolled after giving informed consent. These subjects live in the San Pedro Martir neighborhood of Mexico City, where we have been conducting research on infectious diseases for the past 24 years. Stool, blood, and milk specimens collected from participants are used for diagnosis of CV and RV infections, for determination of antibody responses to the infections, and for determination of the host specificity by characterization of the genetic identity (hiso-blood group antigen and lactadherin expression) of the populations. Children must be studied for both CV and RV infection because age-stratified seroprevalence studies indicate that the peak age for acquisition of antibody for most strains is in the first 5 years of life. All new procedures will be submitted for review through Institutional Review Boards of the Institute of Nutrition in Mexico and Cincinnati CHMC. A summary of the individuals to be enrolled, the time period of enrollment, location of enrollment, the proposed number of enrollees and gender and minority considerations are given below grouped by areas of study.

a) Three hundred six mother-infant pairs followed from the birth of the infant to 24 months of age have been enrolled in the current cohort study in San Pedro Martir, Mexico City. All infants are healthy, full-term infants born without congenital defects who have been breastfed from birth. All of the infants are Hispanic. Infants have been included if the parents gave informed consent, if the infants were born to mothers living in this community, and if the mother planned to breastfeed her infant. Potential subjects were excluded from the study if consent was refused, the mother did not plan to breastfeed, or the infant had either a major congenital malformation or prematurity. All of the infants have been enrolled under IRB reviewed and approved protocols during studies for NICHD PO1 13021 "The role of human milk in infant nutrition and health" (see Appendix IRB approval letters). Status. IRB review will continue with Cincinnati Children's Hospital Medical Center and the Institute's IRB on a yearly basis as long as this study continues.

b) During the 1988-92 grant cycle, 316 mother-infant pairs were enrolled and followed from birth of the infant to 24 months of age in San Pedro Martir, Mexico City. All infants were healthy, full-term infants born without congenital defects who agreed to initiate breastfeeding. All infants were Hispanic and all parents signed an IRB approved informed consent. All continued analyses remains under the IRB approval from all necessary institutions for the current grant cycle and will be reviewed on a yearly basis. IRB approval has been obtained and informed consent will be required to collect an additional sample of saliva and buccal cells from these participants.

Sources of Materials: All research material will be from living human subjects. All blood, stool, milk, and saliva specimens have been and will continue to be coded with a number without personal identifiers before being sent to any laboratory. Clinical information will continue to be collected on mothers and infants so that clinical and epidemiologic data can be related to specimen results. All data will continue to be kept in a secure location.

Potential Risks: The potential risks to mothers and infants associated with the collection of blood, milk, and stool specimens are minimal for subjects enrolled in the proposed projects or specimen collection aspects of the projects.

2. Adequacy of Protection Against Risks

Recruitment and Informed Consent: Research subjects have been recruited in San Pedro Martir section of Mexico City, which has been our study site for the past 24 years. All subjects have signed a consent form following a complete description of the study. All projects and forms have been approved by institutional review boards both at the U.S. and Mexico institutions.

Protection Against Risk: Each participant enrolled in the cohort has been assigned a unique study number that has been used to maintain confidentiality. These unique numbers are used to link specimens with

important data collected weekly by study workers. All specimens sent to laboratories will be coded, to be matched with collected data through the unique study numbers. All identifiable information will be stored in a password protected computer file accessible only to study personnel. All forms pertaining to the results of questionnaires, clinical examinations, and laboratory tests are identified by the study number. Computer data sets generated from such forms also lack identifiable data. Statistical analyses of the study results will be presented in aggregate format in technical reports to the sponsor and in manuscripts submitted for publication in scientific journals. Such reports will not permit the identification of individual participants.

3. Potential Benefits of the Proposed Research to the Subjects and Others

The potential benefits to the subjects include close monitoring of health status with early intervention if illness occurs, parent education about child health, and support for continuation of breast-feeding provided by the study personnel. Since there is minimal risk in collecting samples, the risk benefit ratio is favorable.

4. Importance of the Knowledge to be Gained

Anticipated new knowledge gained concerning CV infection and immunity may greatly benefit other children in the future.

Inclusion of Women Due to the nature of this study of human milk the women and their infants pair are necessarily the subjects of study. Please see the Inclusion Enrollment Format Pages for the actual breakdown of subjects.

Inclusion of Minorities Since one of the major study sites has been established in Mexico, a high proportion of our subjects are Hispanic. Please see the Inclusion Enrollment Format Page for the actual breakdown of subjects.

Inclusion of Children Due to the nature of this study, the inclusion of women and children will be essential. Enrollment for all proposed studies include women and children, as stated in the research and methods section of this proposal. The investigative team has been deeply involved with pediatric healthcare and research for 20 years. The Instituto Nacional in Mexico has been involved with San Pedro Martir for the past 24 years and is revered within this community. The established diarrhea clinic within the community provides facilities to accommodate children. Their dedicated involvement with pediatric healthcare has gained them respect and admiration and their continued involvement is vital, not only to the health of the children, but also to the progression of research. The Cincinnati Children's Hospital Medical Center is fully equipped with appropriate accommodations for all age ranges of children. The years of experience within pediatrics in this institution is vast.

We have carefully considered sample sizes that include a sufficient number of children to contribute a meaningful analysis relative to the purposes of the individual protocols (see research and methods section in the Core section).

F. Vertebrate Animals.

The use of vertebrate animals will be restricted to the production of antibodies and study of virus-host interaction. For production of antibodies, mice will be used to produce monoclonal antibodies. Rabbit and guinea pigs will be used to generate hyperimmune antisera. These monoclonal and hyperimmune antibodies will be used for diagnosis of CVs. Mice also will be used to test naturally occurring and synthetic antiviral compounds and for development of transgenic mouse line expressing human blood group antigens, which is expected after year 3 of the grant period. The transgenic mouse line will be developed using the transgenic mouse Core at the CCHMC.

1. We will use two 6-week-old female Hartley guinea pigs and one pathogen-free rabbit per antigen (recombinant CV capsid antigen). For production of monoclonal antibodies, two Balb/C mice per

antigen will be used. Each animal will be bled for pre-immune serum, serum after three times of immunization, and post-immune serum specimens (6-7 weeks).

2. Hyperimmune antisera can be obtained only from immunized animals and only the hyperimmune antisera are likely to have sufficient affinity and avidity for the proposed studies. Rabbits, guinea pigs, and mice will be given 60-80, 20-30 and 5-10 ug, respectively, of the recombinant capsid antigen per injection and about 150-200, 10-15, and 0.1-0.5 mL of blood, respectively, will be obtained.

3. Animals are housed in a separate Animal Care Facility staffed by veterinarians and care will be provided according to institutional guidelines meeting or exceeding those outlined in the Guide for Laboratory Animal Facilities and Care, from the National Academy of Sciences - National Research Council.

4. Prior to each attempt to draw blood, the guinea pigs and rabbits will be anesthetized with ketamine. Mice will be bled by tail vein and no anesthesia is required. Vein puncture on guinea pigs and rabbits will be performed under the supervising of the school's veterinarian.

5. The animals will be euthanized by injection of ketamine (80-100 mg/kg) and xylazine (50 mg/kg) for guinea pigs and rabbits after bleeding. Mice will be sacrificed by CO_2 asphyxiation. These procedures are consistent with the recommendations of the Panel on Euthanasia of the American Veterinary Medical Association.

Campylobacter Project

A. Specific Aims

Campylobacter is among the most common causes of bacterial diarrhea in children. Infection by pathogenic Campylobacter is inhibited in vitro and in vivo by human milk oligosaccharides. The oligosaccharides responsible for this inhibition contain fucose linked by an α1,2 glycosidic bond. These linkages are produced by the 2-fucosyltransferase of milk that is the product of the secretor gene, *FUT2*. This project is based on the hypothesis that fucosyl α1,2-linked fucose moieties on the host cell surface are essential determinants of Campylobacter infection, and that human milk oligosaccharides containing such moieties are components of an innate immune system of milk that protects the breastfeeding infant from Campylobacter. We propose to determine whether these oligosaccharides also protect against other related enteric pathogens, such as *Vibrio cholerae*; to determine whether natural variation in milk concentrations of the products of the *FUT2* gene are associated with differences in the risk of diarrhea in breastfed infants; and to find the simplest α1,2 linked fucosyloligosaccharides that protect infants from Campylobacter and related causes of diarrhea. To achieve these goals, we propose the following specific aims:

1. Characterize the Campylobacter adhesin associated with binding to host cell receptors.
2. Define the molecular basis for affinity of Campylobacter and *V. cholerae* to α1,2-linked histo-blood group antigen of epithelial cells, and for inhibition of such binding by human milk oligosaccharides.
3. Determine the genetic polymorphisms that underlie maternal and infant secretor and Lewis phenotypes, and relate these to heterogeneity in human milk oligosaccharide expression and risk of bacterial diarrhea in breastfed infants.
4. Measure the safety, tolerance, and efficacy of the human milk oligosaccharide 2'-fucosyllactose, and related compounds, to protect against Campylobacter and related pathogens in mice.

The increased understanding of how human milk oligosaccharides interfere with the pathophysiology of Campylobacter and related pathogens, and of the genetic determinants of heterogenous expression of these oligosaccharides in human milk would allow identification of infants at risk for these pathogens, and potentially lead to novel therapeutic agents that could be used to prevent and treat infections by Campylobacter and related pathogens. These studies are designed to provide the basis for initiating clinical trials on the efficacy of such oligosaccharides against Campylobacter and related diarrhea.

B. Background and Significance

Campylobacter strains are among the most common pathogens in human and veterinary medicine worldwide (1-8). Although diarrhea is the most frequent clinical presentation associated with Campylobacter, a broad clinical spectrum is observed with this infection, including bacteremia, localized infection, and Guillain-Barre Syndrome, a severe immunoreactive complication (1-2). In the United States, the estimated incidence of Campylobacter is two million symptomatic infections per year, approximately 1% of the U.S. population (3). Population-based studies in England, the U.S., and Sweden have shown a bimodal distribution, with a peak of illness in children less than 5 years of age and a second peak in adolescents and young adults 15 to 29 years old (4-8). The highest isolation rate of 15 per 100,000 occurs in the first year of life (5). In endemic areas of developing countries, the isolation rate among children with diarrhea is 8% to 45%, with a similar rate of isolation among asymptomatic children (9,10). The annual incidence of Campylobacter infections can be as high as 2.1 episodes per child-year. Foodborne infections are an emerging concern affecting millions of individuals every year. Campylobacter is the second most common cause of foodborne infection after calicivirus (3-4). The alarming increase in multiple antibiotic resistant strains of Campylobacter being isolated probably results from the use of quinolones in veterinary medicine and as animal food supplements (11).

Adaptive and innate immunity of Campylobacter. The occurrence of Campylobacter diarrhea in young children protects against subsequent infections. Immunity to *C. jejuni* is acquired after one or more symptomatic infections; however, no protection seems to occur after a symptom-free infection (9). Effective natural immunity among children living in endemic areas is the result of an intense early exposure to the organism, as suggested by the high incidence of infection in the first year of life, with progressive decrease in the illness-to-infection ratio with increasing age (9-10). Studies in breast-fed Mexican children have demonstrated protection against Campylobacter diarrhea conferred by specific secretory IgA antibodies present in human milk (12-13). Although immunoglobulins in milk provide important protection against Campylobacter as well as other causes of respiratory and gastrointestinal tract infections (14-16), non-immunoglobulin components also appear to play an important role (17-19). Among the non-immunoglobulin protective factors in human milk, the oligosaccharides and glycoconjugates appear to be the most important (19-21). However, the specific glycoconjugates and oligosaccharides involved in protection against specific causes of infectious illness are not yet well defined. Likewise, the mechanisms by which the oligosaccharides interact with mucosal pathogens to prevent or reduce the severity of infections are not well understood. Our data indicate that the initial steps of attachment of Campylobacter to the host cell surface, critical to infection, involve binding to epithelial cell surface glycoconjugates (20,22). Human milk oligosaccharides with structural homology to these ligands may inhibit binding by the pathogen (22). Therefore, variable expression of these oligosaccharides in milk due to maternal genetic heterogeneity may influence the risk of infection in breastfed infants (23-25).

Fucose tropism of Campylobacter. Recent advances in understanding the pathogenesis of Campylobacter infection have followed the sequencing of its complete genome (26). Nevertheless, the intimate mechanisms of interaction with host cells are not well understood. The ability of Campylobacter to adhere to and invade the epithelial cells of the ileum and cecum is well known (27-32). Motility and chemotaxis play a major role in the localization of bacteria in the lower part of the intestine (33-37). Studies of the chemotactic behavior of Campylobacter have shown a positive response to the presence of fucose, but not other sugars, as well amino acids such as aspartate, cysteine, glutamate and serine (35). L-Fucose is an important constituent of both bile and mucin. These may be important factors for the affinity of the organism for the gall bladder and the lower intestinal tract. Environmental and chemotactic stimuli specifically upregulate the *Campylobacter jejuni fla*A sigma 28 promoter (36). High pH, osmolarity, and bile salts, including deoxycholate, also upregulate the *fla* promoter while high viscosity results in downregulation of the *fla* promoter. Considering that bile and mucin are mixed together in the intestine, and that *C. jejuni* colonization of the mucin layer is a prerequisite for pathogenesis in vivo, the overall response would be an increase in *fla*A synthesis and chemotaxis towards the mucin layer. These data explain the importance of fucose in the pathogenesis of Campylobacter infection in the gastrointestinal tract. From our cohort studies, we have isolated Campylobacter strains from asymptomatic children that do not adhere to or invade cells in vitro and do not colonize experimental animals (9,20). Furthermore, these strains can be differentiated from invasive strains by RAPD fingerprinting or by amplification of the *iam* locus, a genetic marker of Campylobacter invasion (38). In recent studies on motility of these strains, we have observed that none of these strains are motile, supporting the importance of motility in pathogenesis. It is important to further characterize these strains to determine whether they have mutation or deletion within the *fla* locus including the *fla* promoters (36). It is also important to define how the *iam* locus participates in the different events of gut colonization, including adherence to and invasion of, intestinal cells. Studies on chemotaxis and the use of microarray technology could help us to define functionally and genetically the mechanism of the selective affinity of Campylobacter for fucose residues (39).

Interaction between Campylobacter, host cell ligands, and human milk. Once Campylobacter has approached the ileum and cecum mucosa, it adheres to and invades epithelial cells by a complex process in which multiple factors participate, such as capsular polysaccharides, particular ganglioside mimicry motifs in the lipopolysaccharide, plasma encoded type IV secretion system, protein adhesins, and other less well defined factors (29,40-42). A genetic locus from Campylobacter has been characterized that appears to be involved in glycosylation of multiple, soluble, and membrane-associated proteins, some of them involved in adherence of epithelial cells (43-44). Specific site mutations of some of the *pgl* genes significantly reduced adherence to and invasion of intestinal cells and significantly reduced the ability to colonize the intestinal

tract of mice, suggesting a role for the general protein glycosylation system in virulence of Campylobacter (45).

Early studies demonstrated inhibition of Campylobacter adherence to intestine epithelial cells by L-fucose (46). We had shown inhibition of cell adherence in vitro and colonization to gut mucosa in vivo by fucosylated oligosaccharides from human milk (21). Characterization of these milk carbohydrate residues showed that α1,2 fucosylated oligosaccharides are the main active components. Thus, fucosylated oligosaccharides of human milk, possibly those containing H-2, Lewis y or Lewis b epitopes, can inhibit Campylobacter adherence to its host receptor. The specificity of binding to α1,2 fucosyl moieties was confirmed by transfecting CHO cells with the human gene for human α1,2 fucosyltransferase whose expression product catalyzes the final step of H antigen synthesis (47). While parental nontransfected CHO cells (which do not express H antigen) are not infected with invasive Campylobacter, transfected cells are susceptible to adherence and invasion by Campylobacter. The differential expression of blood group antigen H-2 at different sites of the gastrointestinal tract could explain the essential features of the pathology of Campylobacter diarrhea, and likewise, the localization of infection. Mice transfected with the *FUT1* gene, flanked by the murine whey acidic protein promoter, specifically express *FUT1* in milk during lactation (48). These transfected mice produce large amounts of H-2 antigens in milk, whereas the wild type mice produce none. Pups nursing these transfected dams were protected against intestinal colonization by Campylobacter. These data support the concept that H antigens are the intestinal ligands essential for the binding of Campylobacter to the intestinal tract. In milk, soluble ligands containing H-2 epitopes can serve as receptor analogs that protect infants from Campylobacter infection, and they may represent an important component of the innate immune system of human milk (19). However, greater understanding is needed of intestinal carbohydrates and glycoconjugates in the pathogenesis of Campylobacter infection, the specific mechanisms of interaction between bacterial surface structures (adhesins, surface glycoproteins) and host cell surface carbohydrate ligands, and the inhibition of such binding by milk glycoconjugates.

Polymorphisms of secretor (*FUT2*) and Lewis (*FUT3*) genes may influence differential susceptibility to gastrointestinal infection. The expression of Lewis and H antigens in mucosal secretions is regulated genetically (23-25, 49). Individuals who lack the expression of ABH antigens in saliva and other mucosa are considered nonsecretors. The presence of H antigens in mucosa depends on the expression of the *FUT2* gene, while the expression of this antigen in erythrocytes depends on the *FUT1* gene (50). Lewis a and b antigens are regulated by *FUT3* (51). These antigens are synthesized in the epithelial cells of the digestive tract and are expressed in blood and secretions such as saliva and milk. Red blood cells absorb the glycolipids carrying the Lewis antigens in plasma; their phenotypes are defined in the erythrocytes by hemagglutination tests. The Le(a-b+) and Le(a-b-) phenotypes of erythrocytes are usually in accordwith the secretor status in mucosal secretions, whereas the Le(a+b-) phenotype correlates with nonsecretor status. However, inconsistency between erythrocyte phenotypes and secretor status is sometimes observed. H antigens expressed in mucosa are the products of α1,2 fucosyltransferases that synthesize both H type 1 and H type 2 structures. Nonsecretor individuals have a nonsense mutation in the *FUT2* gene that totally inactivates the gene, such as the G428A mutation frequently found in Caucasians, or a missense mutation that partially inactivates the gene, such as the A385T mutation frequently found in Asian populations (49,51). Therefore, individuals homozygous for nonfunctional alleles of the secretor gene (se/se) fail to express Lewis histo-blood group antigens in secretions. This lack or partial expression of the H antigen in mucosal secretions and milk may define much of the variation in susceptibility to gastrointestinal infections in different populations (52). Recent studies of the polymorphisms of the Lewis and secretor genes have demonstrated differing susceptibility to helicobacter infection (25). Ikehara et al reported that *H. pylori* infection rates were associated with genotype: The infection rate was positively associated with the number of Se alleles, with a significantly higher rate of *H. pylori* infection in the Se/Se genotype as compared with the se/se. The infection rate was negatively associated with the number of Le alleles in which *le/le* genotype was associated with higher infection rate as compared with Le/Le. The highest risk was seen in the Se/Se, le/le genotype (adjusted OR 10.2) and the lowest with the Le/Le, se/se genotype (adjusted OR 1.0). Our recent studies support this concept of susceptibility to enteric infection being dependent on the expression of *FUT2* in secretions, with the greatest protection conferred on breastfed Mexican children whose maternal milk contains the highest amounts of α1,2-linked fucosyloligosaccharides (53). Infants

consuming milk with high levels of oligosaccharides containing H-1, H-2, and Lewis b epitopes had a significantly lower risk of moderate to severe diarrhea. High concentrations of Lewis b in milk were associated with protection against diarrhea due to calicivirus. High levels of H-2 in milk were specifically associated with protection against infant diarrhea due to Campylobacter.

Significance. The major hypothesis of this project is that a critical step in Campylobacter pathogenesis requires binding to a 2-linked fucosylated host cell surface receptor. Human milk 2-linked fucosyloligosaccharides would inhibit this binding due to homology between the milk oligosaccharide and the host cell surface receptor. Other related pathogens that share this receptor specificity, like cholera, would also be inhibited by the same milk oligosaccharides and the same mechanism. This inhibition by oligosaccharides could also involve functioning as a chemotactic effector, redirecting Campylobacter motility from the mucosal epithelial cells to the lumen of the gut. Further, glycosylated proteins on the surface of Campylobacter itself may contribute toward colonization and cell adherence; milk oligosaccharides could interfere with this type of binding as well.

A better understanding of the oligosaccharide structure of H-2 cell receptors of *C. jejuni* and *V. cholerae* could provide a target for vaccine development, and will assist with the selection of the simplest fucosyloligosaccharide that is most effective at inhibiting Campylobacter and related pathogens. Synthetic oligosaccharides that are effective inhibitors of these pathogens could represent a novel class of selective and potent new prophylactic and/or therapeutic agents. Defining the genetic basis for heterogenous expression of these oligosaccharide epitopes, both in the intestine of the infant, where they would confer risk, and in milk, where they would protect against this risk, would allow us to understand host specificity for these pathogens. This would make possible the definition of populations and individuals at risk for these pathogens who might benefit most from supplementation with these novel protective oligosaccharides. Figure 1 provides a visual representation of the conceptual model that guides our research.

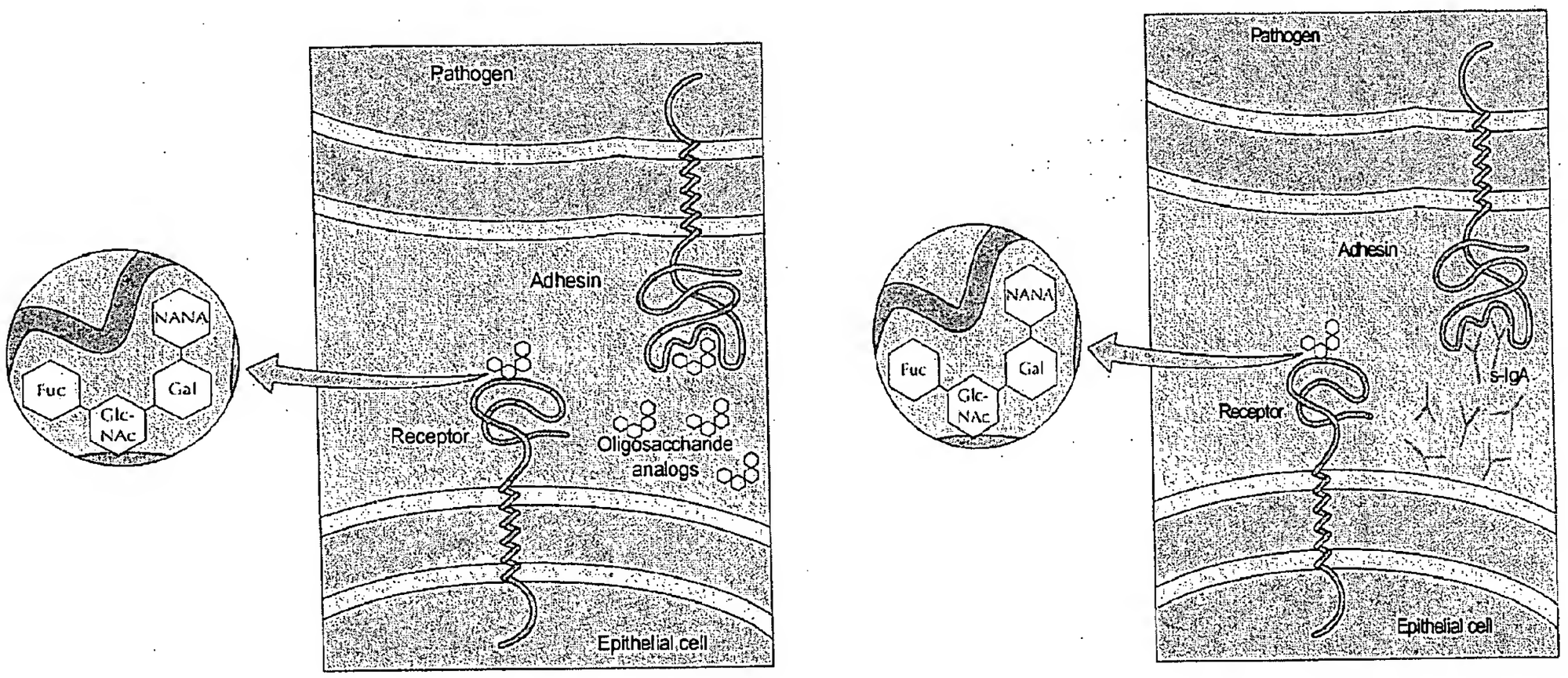


***Figure 1**: Theoretical model. Inhibition of bacterial binding by either milk or synthetic glycoconjugates or by specific antibodies induced by immunization with bacterial antigens that specifically bind to fucosyl ligands of intestinal cells.*

C. Progress Report

During the current grant cycle our specific aims have been to: 1) define the virulence markers of Campylobacter associated with adherence to and invasion of epithelial cells; 2) further characterize and purify milk receptor glycoconjugates that inhibit Campylobacter colonization and infection; 3) assess passive protection against Campylobacter infection conferred by fucosylated milk glycoconjugates in breastfed children and in transgenic mice carrying the human fucosyltransferase gene; and 4) develop strategies for large-scale synthesis of fucosylated glycoconjugates active against Campylobacter infection. Our progress on each of these aims is described below.

Specific Aim 1. Define the virulence markers of Campylobacter associated with adherence to and invasion of epithelial cells.

Characterization of invasive and non-invasive Campylobacter. Adherence to and invasion of epithelial cells are the most important pathogenic mechanisms of Campylobacter diarrhea. During this grant period, we studied the molecular characteristics of invasive and non-invasive *Campylobacter jejuni* and *Campylobacter coli* isolates (38). Molecular characterization of invasive Campylobacter isolates from children with diarrhea and noninvasive strains from symptom-free children was performed by random amplified polymorphic DNA techniques (RAPD). A distinct RAPD profile with a DNA band of 1.6 kb was observed significantly more frequently in invasive (63%) than noninvasive (16%) Campylobacter isolates ($p<0.0001$). The 1.6 kb band was named the invasion-associated marker (*iam*). Using specifically designed primers, a fragment of 518 bps of the *iam* locus was amplified in 85% of invasive and 20% of noninvasive strains ($p<0.0001$). Molecular typing with a PCR restriction fragment length polymorphism assay that amplified the entire *iam* locus showed a *Hin*dIII restriction fragment polymorphism pattern associated mainly with invasive strains. Although cluster analysis of the RAPD fingerprinting showed genetic diversity among strains, two main clusters were identified (see Figure 2, next page). Cluster I was comprised of significantly more pathogenic and invasive isolates than cluster II, which grouped the majority of nonpathogenic, noninvasive isolates. These data indicate that the majority of invasive Campylobacter strains could be differentiated from noninvasive isolates by RAPD analysis and PCR using primers that amplify a fragment of the *iam* locus.

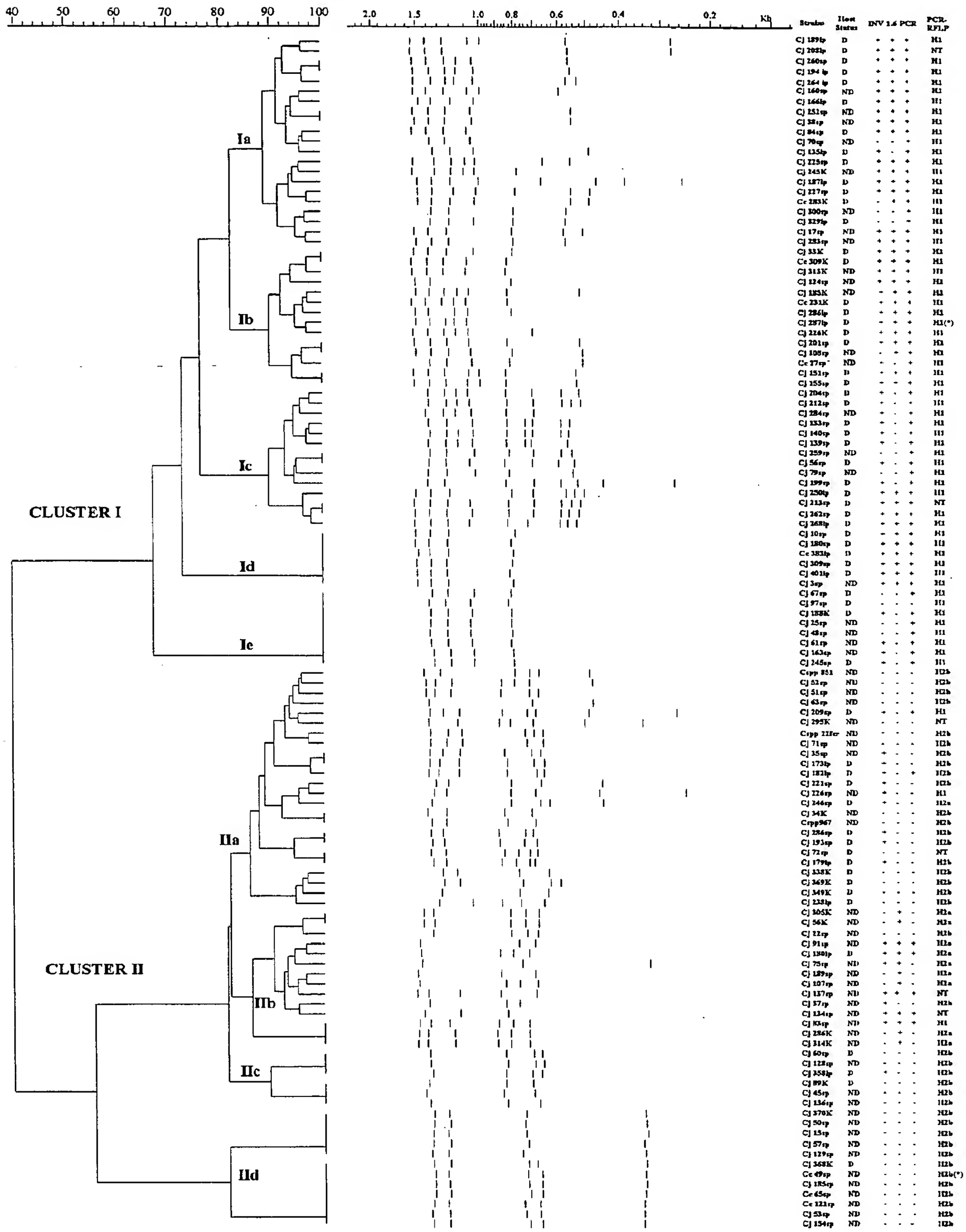


Figure 2: *Oligonucleotide 1290 RAPD fingerprinting dendogram shows cluster analysis results of 119 invasive and non-invasive Campylobacter strains. On the right side of the figure are columns describing strain denomination, host status, invasive phenotype, presence of iam, PCR amplification of iam locus, and PCR-RFLP pattern.*
D, diarrhea; ND, no diarrhea; +, positive; -, negative.

To confirm the importance of the *iam* locus on adherence and invasion, we used insertial knockout mutants of *iam* A, B, and C using a chloramphenicol-resistant cassette constructed in our laboratory for this particular 287 *C. jejuni* strain. Cell invasion was reduced to less than 10% and studies of colonization in mice demonstrated 84% reduction in colonization (manuscript in preparation) (Figure 3). Since few non-invasive strains possessed the *iam* locus, we hypothesize that there may be important differences in the sequence of *iam* in these strains when compared with the invasive strains. The nucleotide sequence of the *iam* locus of our reference invasive strain 287-IP and the non-invasive strain 50-SP was determined from phagemide clones obtained from genomic libraries constructed in λ Zap. A907-bp fragment of the *iam* locus was amplified and sequenced from the 11 non-invasive *iam*-positive strains. All sequences were compared with the reported sequence of reference strain 11168 (www.sanger.ac.uk/projects/C_jejuni). Noninvasive strain 50-SP and reference strain 81176 with a low invasive index had 100% homology with the reported

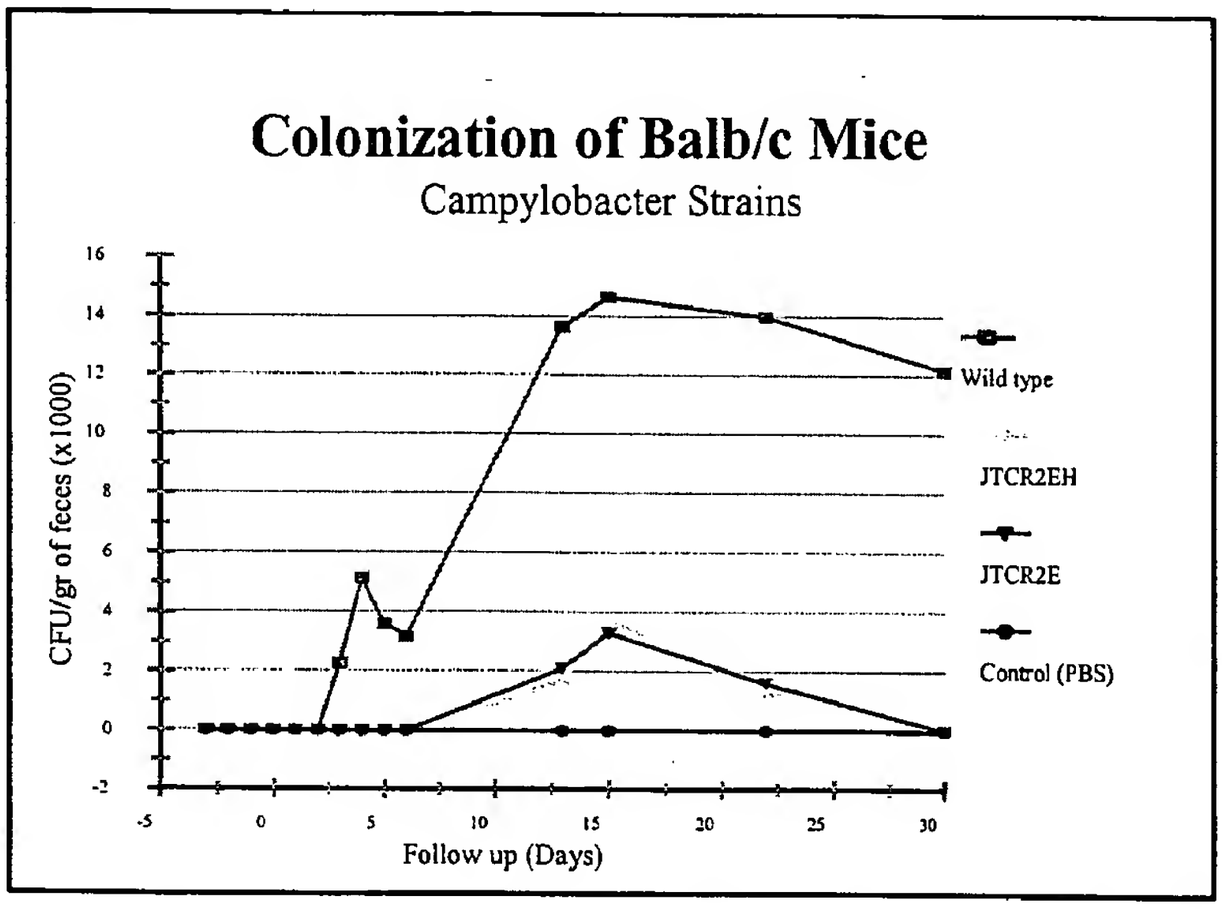


Figure 3: *Colonization of Balb/c mice with wild 287-IP invasive strains of Campylobacter (upper line marked by squares), and in mice colonized with the iam:: cat mutants (lines marked by triangles).*

sequence of strain 1168; the highly invasive strain 287-IP had 86% homology. Surprisingly, all 11 of the non-invasive PCR-positive strains had 100% homology with strain 287-IP, suggesting that, although they possess the complete *iam* locus, there may be another mechanism involved in regulating the ability of Campylobacter to invade. The use of microarray technology may help us analyze and identify other genes involved in Campylobacter adherence and invasion. We plan to develop and apply microarray technology in the proposed grant period (39).

Identification of Campylobacter adhesin. We previously observed that supernatants of *C. jejuni* suspension with deoxycholate induce agglutination of HEp-2 cells. To further characterize this deoxycholate-induced agglutinin, a suspension of a *C. jejuni* culture was incubated with deoxycholate and concentrated by ultracentrifugation. The extract was subjected to continuous-elution electrophoresis, and the fractions were dialyzed and tested in HEp-2 cells agglutination assays (Figure 4a). The fractions that clearly induced agglutination were those showing a band of 55-57 kDa (Figure 4b). We are now sequencing this protein.

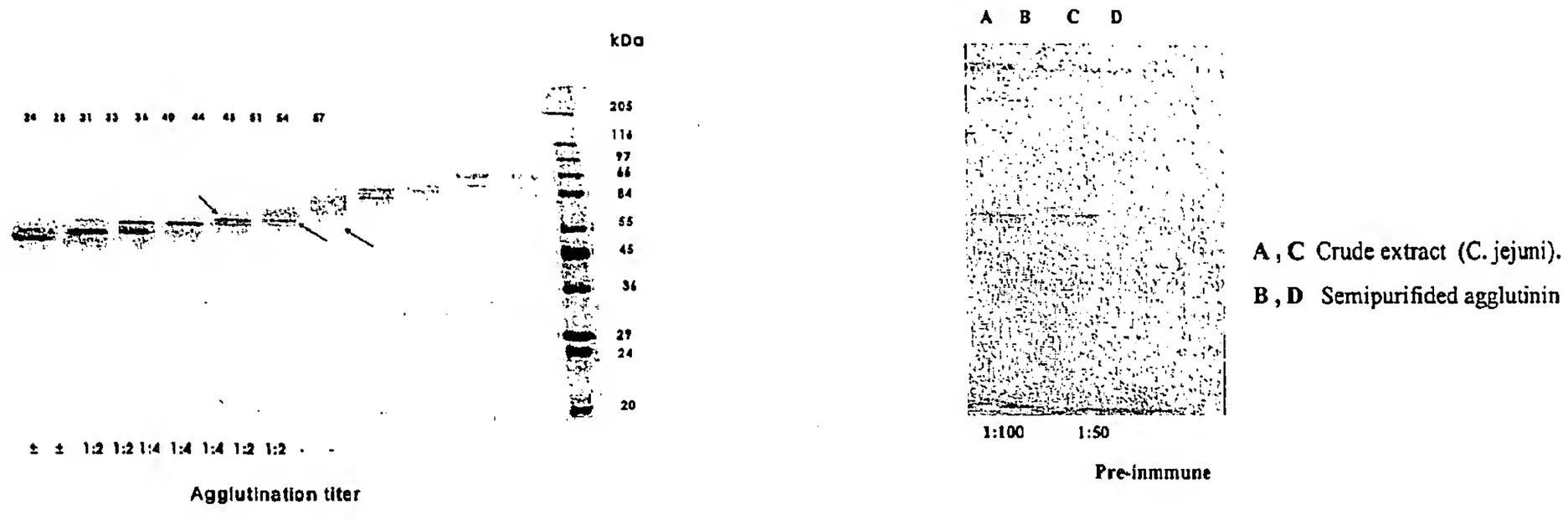


Figure 4a: *Partial purification of the deoxycholate induced Campylobacter jejuni cell agglutinin by continuous-elution electrophoresis. The arrows mark a 55-57 kDa protein with a high cell agglutination activity.*

Figure 4b: *Western blot of the 55-57 kDa agglutinin using a rabbit hyperimmune sera.*

Molecular tools for studying Campylobacter-host cell interaction. A major advance during the current funding cycle was the development of molecular tools to further study the interaction of Campylobacter with milk oligosaccharides and host cell surface ligands. First, we constructed a shuttle plasmid called pstr008.12 that carries a fluorescent protein (gfp) gene of *Aequorea victoria* and a chloramphenicol resistant (cat) gene designed to make stable fluorescent constructs which retain the plasmids. Further, we developed plasmids that can be used for expression and site mutation in strains that are multiply antibiotic resistant, a common feature in Mexican strains. This development is important to our work and that of others conducting research in areas of the world with high background rates of antibiotic resistance in pathogenic bacteria.

Specific Aim 2. Further characterize and purify milk receptor glycoconjugates that inhibit Campylobacter colonization and infection.

Characterization of milk receptors that inhibit Campylobacter adherence. During the current grant cycle, we established that the specific binding of Campylobacter in HEp2 cells is inhibited by fucosylated carbohydrate moieties containing the H(O) blood group epitope (Fucα1,2Galβ1,4GlcNAc). Studies of Campylobacter binding to histo-blood group antigens as neoglycoproteins immobilized in nitro-cellulose membranes demonstrated a high avidity for the H-2 antigen as confirmed by specific inhibition with monoclonal antibodies.

In studies on the mechanism of adherence, *Campylobacter jejuni*, which normally does not bind to Chinese hamster ovary (CHO) cells, bound avidly when the cells were transfected with a human α1,2 fucosyltransferase gene that caused overexpression of H-2 antigen (Figure 5). Similarly, *V. cholerae* adheres to transfected (Figure 6) but not to parental cells (data not shown).

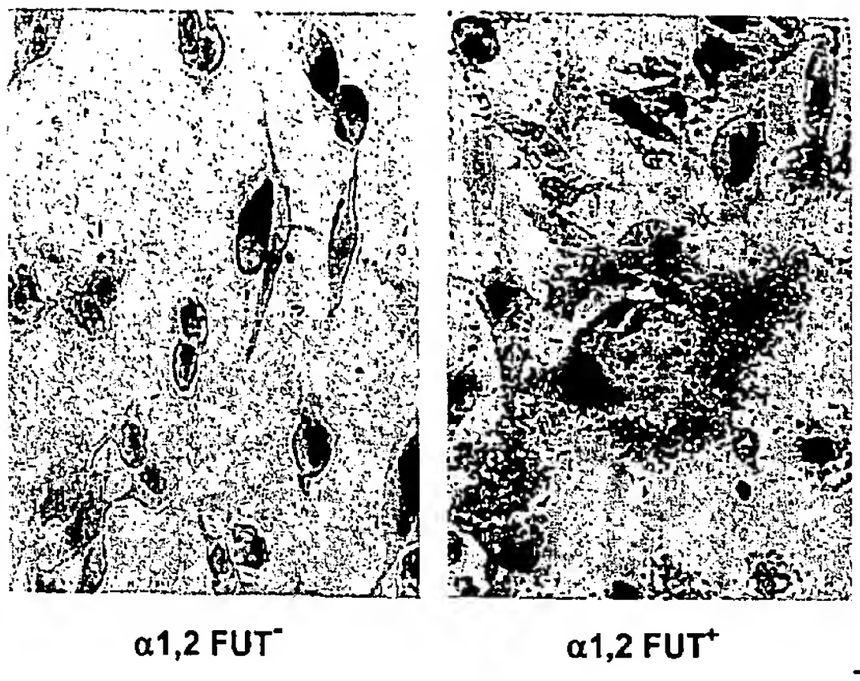


Figure 5: *Campylobacter jejuni binding to parental CHO cells carrying the plasmid vector only (right) and binding to FUT1(α1,2 fuc) transfected CHO cells (left).*

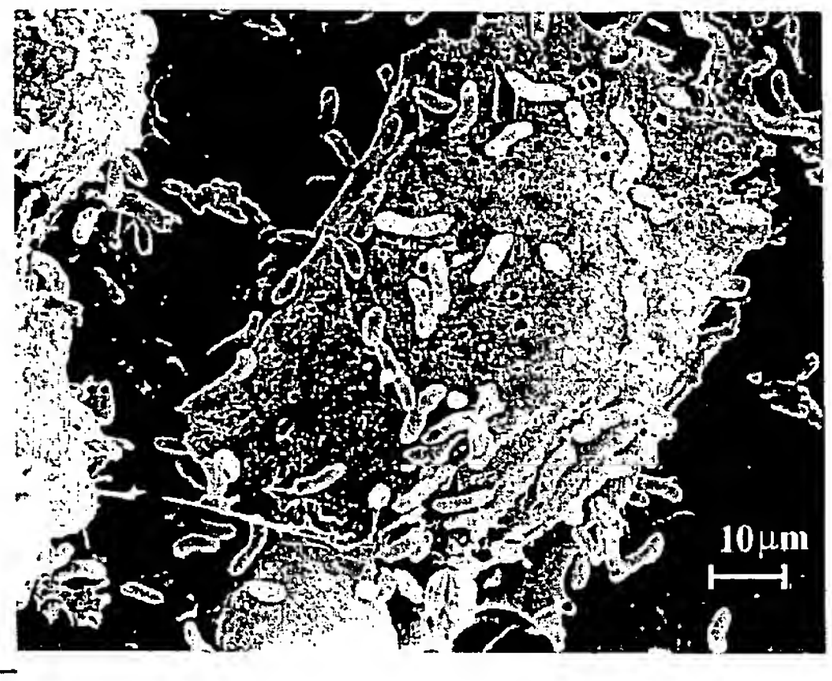


Figure 6: *Scanning microscopy V. cholerae binding to FUT1 transfected CHO cells.*

This binding was specifically inhibited by H-2 ligands (*Ulex europaeus* lectin, Lotus tetragonolobus lectin, and H-2 monoclonal antibody), H-2 mimetics, and human milk oligosaccharides (Figure 7). Invasive Campylobacter 287-IP binds to *FUT1*, but not *FUT3* or *FUT4*-transfected CHO cells (Figure 8).

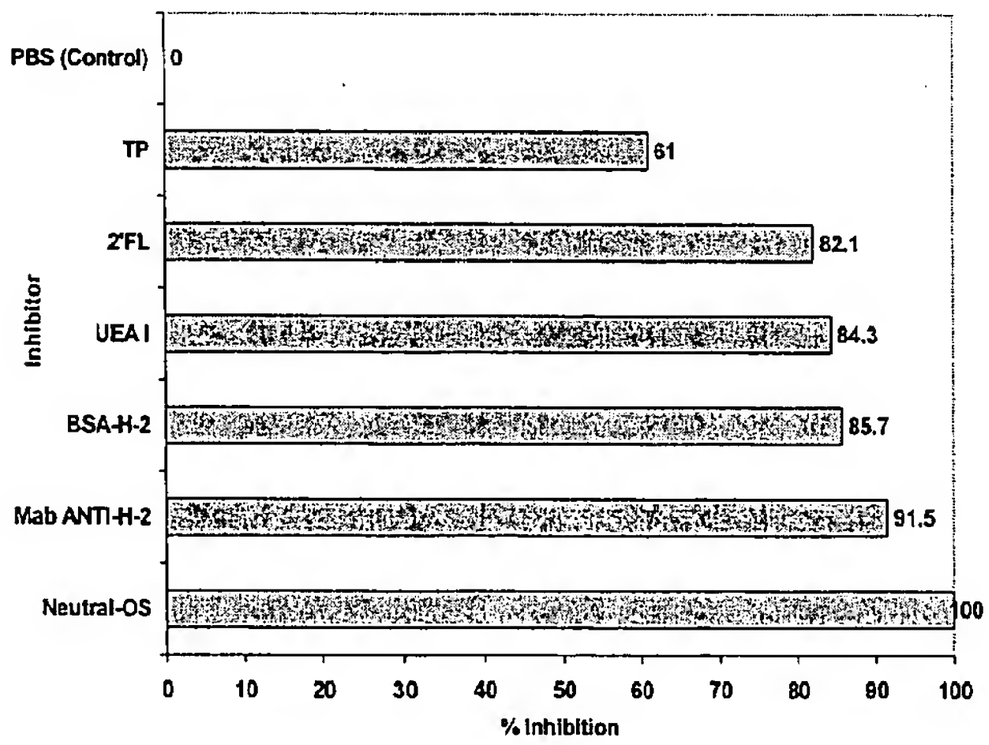
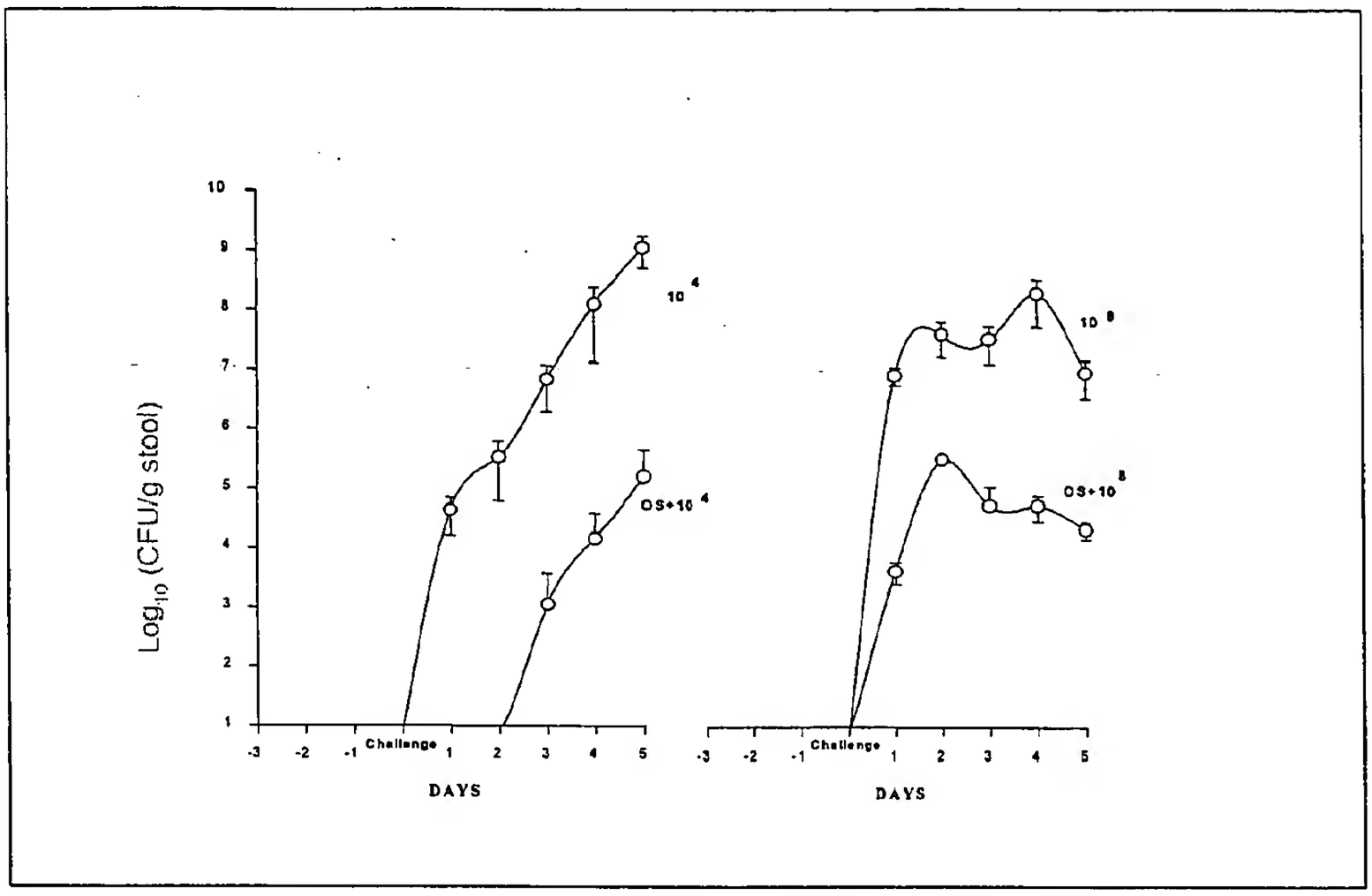


Figure 7: *Inhibition of Campylobacter binding to FUT1- CHO cells by H-2 ligands and H-2 mimetics and human milk oligosaccharides.*

Cells	Pathogenic					Non Pathogenic	
	UEA I	INN 287IP	INN 84SP	INN 166IP	NN 10SP	INN 50SP	INN 57SP
FUT1 (α1,2)	3+	2+	2+	1+	2+	0	0
FUT3 (α1,3)	0	0	0	0	0	0	0
FUT4 (α1,3 and α1,4)	0	0	0	0	0	0	0
Parental CHO	0	0	0	0	0	0	0

Figure 8: *Cell agglutination induced by invasive Campylobacter strain 287ip on transfected CHO cells carrying FUT1 (1,2 fuc), FUT3 (1,3/1,4), and FUT4 (1,3 fuc) gene.*

In experimental models, human milk oligosaccharides inhibited Campylobacter colonization in mice *in vivo* and in human intestinal mucosa *ex vivo*. (Figure 9).



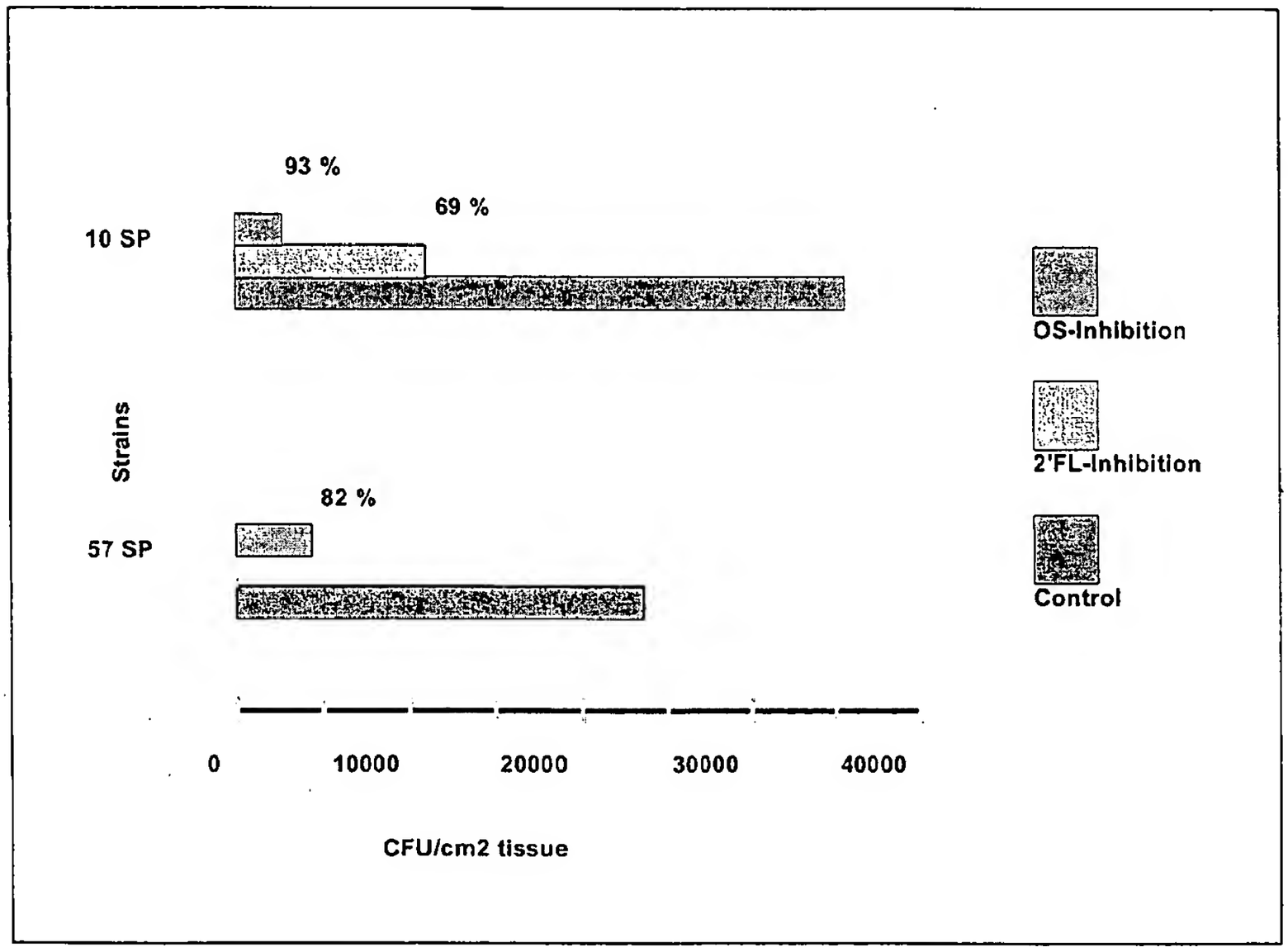
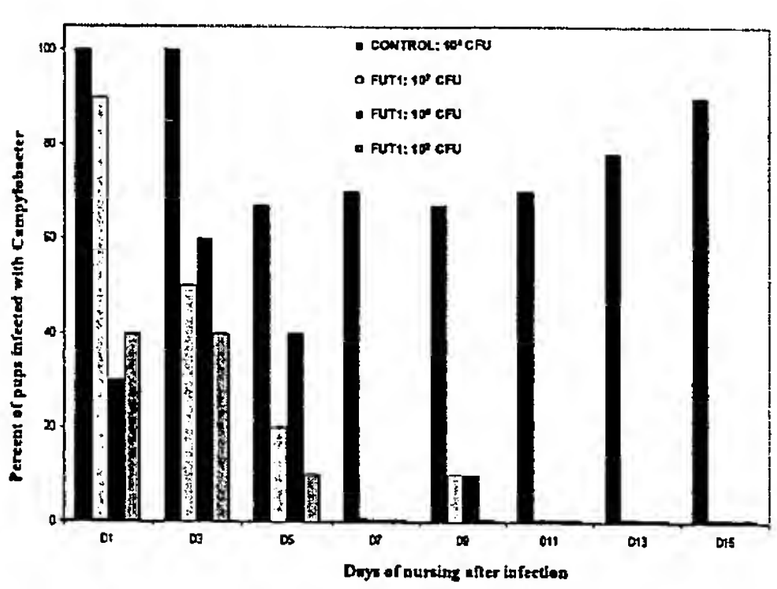


Figure 9: *Inhibition of Campylobacter colonization in BALB/c mice fed with 2 mg of milk fucosylated oligosaccharides given during challenge with 10^4 and 10^8 CFU of bacteria (left). Ex vivo assays of inhibition of human gut colonization of Campylobacter with 2'-fucosyllactose (2'-FL) and milk fucosyleted oligosaccharides (OS).*

Transgenic mice. The role of milk α1,2 glycoconjugates in passive protection against Campylobacter infection was evaluated in litters of B6-SJL transgenic female mice carrying the human α1,2 fucosyltransferase gene (*FUT2*) with a whey promoter that induces the expression of histo-blood group antigens primarily in mammary gland during lactation, and thus, in milk. As a control, non-transgenic parental mice were used. Suckling mice were challenged with 10^4, 10^6 and 10^8 CFU of *C. jejuni* and were returned to the dams. Gut colonization was monitored for 15 days. Up to 90% of non-transgenic litters remained colonized during follow-up. Colonization of transgenic mice was transient and the time of colonization was directly related to the inoculum (Figure 10). These experiments strongly support the role of α1,2-linked fucosylated glycoconjugates of milk in protection against Campylobacter infection, and suggest that the main intestinal ligands for Campylobacter are the H-2 histo-blood group antigens. Milk fucosyloligosaccharides and specific fucosyl α1,2-linked molecules inhibit this binding and may represent a novel class of antimicrobial agents. These results are submitted for publication.



Days after Infection	Transgenic (FUT1)			Non-Transgenic
	Campylobacter inoculum (CFU/ml)			
	x10⁴	x10⁸	X10⁹	Control x10⁸
1	40	30	90	100
3	40	60	50	100
5	10	40	20	67
7	0	0	0	70
9	0	10	20	67
11	0	0	0	70
13	0	0	0	78
15	0	0	0	88

Figure 10: *Campylobacter colonization in transgenic mice carrying* the *FUT1 gene with the WAP promoter that directs the expression of H antigens primarily to lactating mammary gland. Pups fed from transgenic mice cleared colonization 5 to 9 days after challenge with Campylobacter. Control pups from non transgenic mice are unable to clear Campylobacter colonization. CFU=colony forming units.*

Immunoassays for detection and quantitation of H-2 glycoconjugates in milk. To determine the secretor status of mothers through testing their milk, a Western blot (WB) assay was developed using the lectin *Ulex europaeus* conjugated with peroxidase to detect H-2 glycoconjugates (α1,2 linked structures) (Figure 11). Milk concentrations of these glycoconjugates were determined by a competitive enzyme-lectin immunoassay (EIA) developed in our laboratory. A curve was standardized with known concentrations of H-2 neoglycoprotein. A total of 139 breast milk samples obtained from Mexican mothers on day 30 of lactation were tested. A strong correlation was observed between the sensitivities of the two assays to identify non-secretors. In this population, 3/139 (2.2%) were WB- and ELISA-negative, corresponding to non-secretors. The distribution curve of H-2 glycoconjugate concentration in milk is shown in the righthand panel of Figure 11.

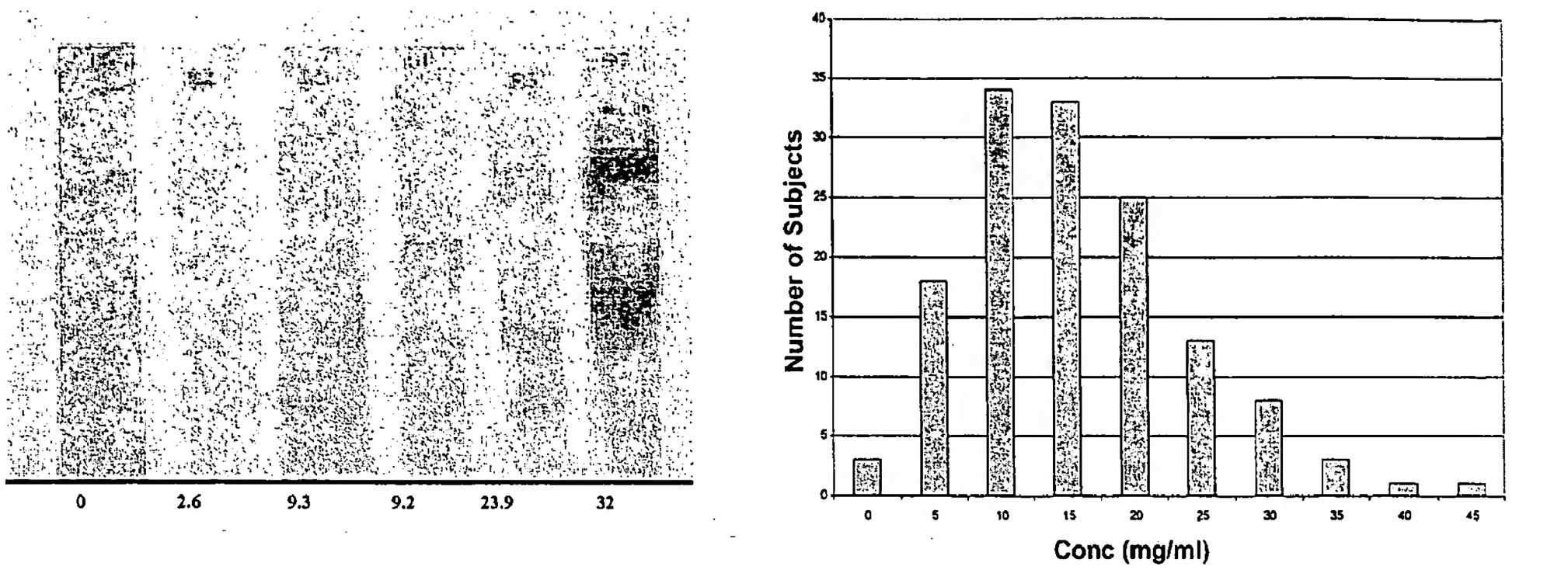


Figure 11: *Expression of H antigens in milk. UEA-1 lectin-Western blot of milk samples from Mexican mothers with increasing concentration of α1,2 glycoconjugates (lefthand panel). Concentration of H (α1,2 fuc) antigen in milk (measured as mg/mL) taken at one month postpartum from 139 breastfeeding Mexican mothers as measured by a competitive EIA (righthand panel).*

H-2 concentrations in milk differed between individuals; among the 139 study mothers at least three groups could be identified: 1) 3 (2.2%) non-secretors; 2) 18 (13%) partial secretors, producing less than 5 mg/mL; and 3) 118 (85%) secretors, producing 5 or more mg/mL of 2-linked oligosaccharides. The genetic characterization of these groups are proposed in the grant cycle covered by this application (2003-2008).

Preliminary characterization of the polymorphism of the *FUT2* gene. During the past year we initiated investigation into the polymorphisms of the *FUT2* loci in our Mexican study population. DNA was obtained from the cellular fraction of milk. Because of the lack of published data characterizing the distribution of fucosyltransferase genes and their mutations in Hispanic populations, we first examined the frequency of the two most common mutations found in other populations, ie, the G428A mutation (found predominantly in individuals of European ancestry), and the A385T mutation (found predominantly in individuals of Asian ancestry) (52). We began by identifying individuals whose phenotype was consistent with being non-secretors and/or partial secretors. From the three non-secretors we identified two who were homozygous for the G428A mutation and one who was heterozygous for this mutation suggesting that there must be another mutation in this individual different from the G428A mutation (Figure 12).

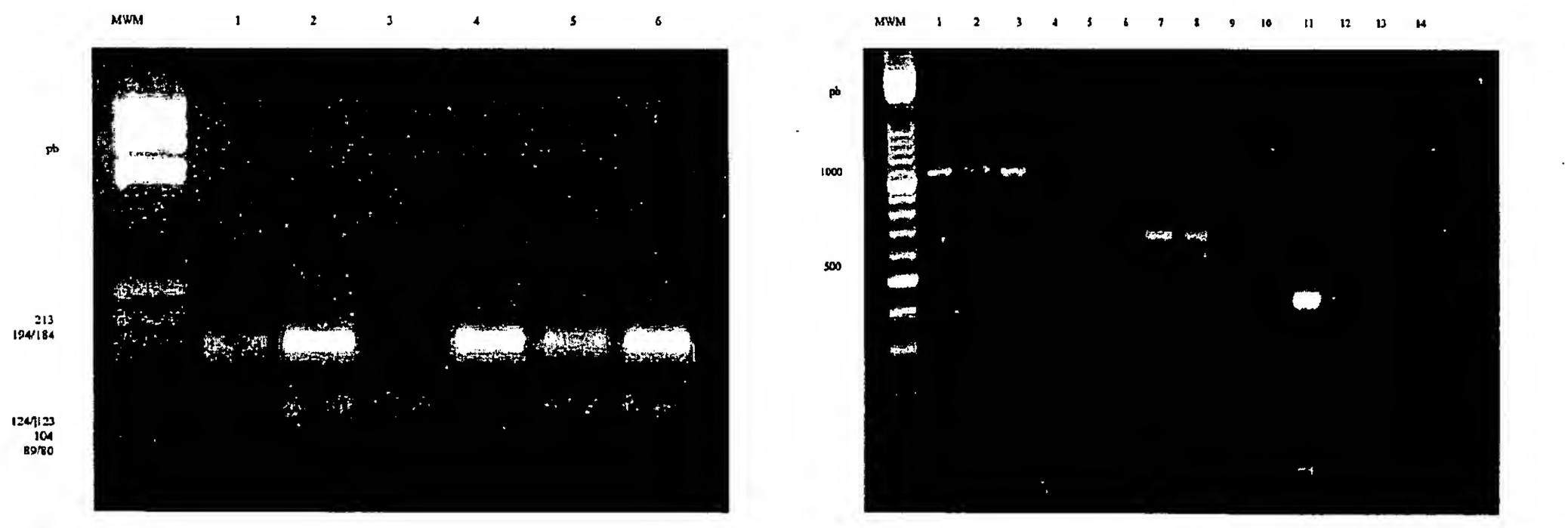


Figure 12: *PCR screening for mutation A428T and A385T of FUT2 gene. The lefthand figure shows the 428 nonsense mutation of homozygous (lanes 1 and 4), heterozygous (lanes 2, 5 and 6), and wild allele (lane 3). The righthand figure shows the 385 missense mutation (lanes 9 and 11).*

At present, we are cloning and sequencing the entire *FUT2* gene to identify other possible mutations in this Mexican population that totally inactivate enzyme activity. The prevalence of heterozygotes for this mutation in partial secretors was 28% (5 out of 18). We have established the PCR technique to identify the frequency for the A385T mutation. At present we have tested 20 mothers, but none have been identified with this mutation. We are planning to test all mothers of this cohort for this mutation. However, our preliminary data suggest that the A385T mutation is not common in the Mexican population. Thus, we are pursuing the possibility that mutations other than 428 or 385 may explain the partial secretors in this Mexican population.

Specific Aim 3. Assess passive protection against Campylobacter infection conferred by fucosylated milk glycoconjugates in breast-fed children and transgenic mice carrying the human fucosyltransferase gene.

Br astfed children. During the current grant cycle we selected a subgroup of 93 breast-feeding mother-infant pairs from the 1988-1992 cohort to examine protection against Campylobacter infection associated with the α1,2-linked fucosylated milk oligosaccharides. The data from these mother-infant pairs included the follow-up time from birth to the end of breastfeeding. A single milk sample from each of these mothers, collected at 1-5 weeks postpartum, was sent to Dr. Newburg's laboratory for analysis by HPLC. The profile of milk oligosaccharides was examined in relation to maternal Lewis blood group type, and in relation to risk of infant diarrhea due to Campylobacter and diarrhea from all causes. In the 93 study mothers, Lewis blood group type distributions were as follows: 67 (72%) were Le (a-b+); 24 (26%) were Le (a-b-) and 2 (2%) were Le (a+b-). Of the 93 study children, there were 31 cases of symptomatic Campylobacter infection during the breastfeeding period. As shown in the Core progress report and the ST project, the fucosylated milk oligosaccharide phenotype differed by maternal Lewis blood group type. The most common milk oligosaccharide in all study mothers was found to be 2'-fucosyllactose (2'-FL), the oligosaccharide homolog of the Lewis H-2 epitope. The mean concentration of 2'-FL in maternal milk samples was 3854 $\pm$ 108 nmol/mL (34% of total fucosylated oligosaccharides). When the individual fucosylated milk oligosaccharides were examined as a percentage of total oligosaccharide, many were negatively correlated with 2'-FL as a percentage of total oligosaccharide (r=-.5 or less). 2'-FL as a percentage of total oligosaccharide was found to be significantly associated with protection against Campylobacter diarrhea (Poisson regression beta coefficient=-5.6 [SE=1.9], p=.004) while LDFH-I oligosaccharide (a Le^b homolog) as a percentage of total oligosaccharide in maternal milk was associated with significantly (p=.047) increased risk of Campylobacter diarrhea. However, when these milk oligosaccharides were taken together in the same statistical model, only 2'-FL (H-2) as a percentage of total oligosaccharide remained significantly associated with Campylobacter diarrhea.

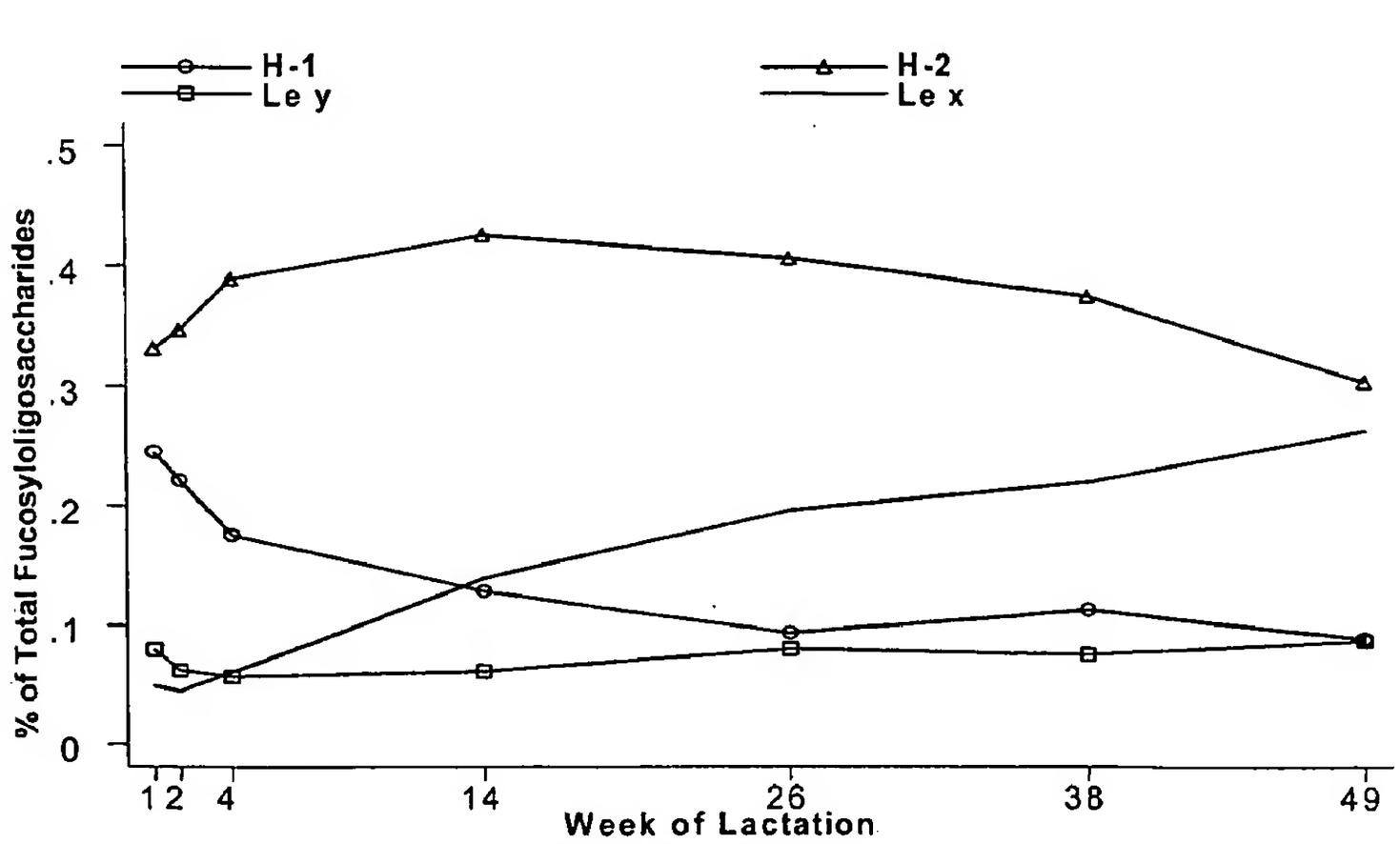


Figure 13. *Longitudinal pattern of four major oligosaccharides of human milk measured in 11 secretor mothers from the Mexico study cohort (1988-1992 cohort). Each line represents the percentage of total fucosylated oligosaccharide due to a specific oligosaccharide Lewis epitope indicated in the figure legend.*

Longitudinal and between-population variation in fucosylated oligosaccharides. Over the past four years, we have collaborated with the Glycobiology Core and others to examine the pattern of fucosylated milk oligosaccharides within our study population in Mexico and to compare the milk samples of Mexican mothers with samples collected from other populations (55,56).

We examined the milk of 11 secretor study mothers from the 1988-1992 cohort who had samples collected from 1 to 49 weeks postpartum (Figure 13 above). We found a dynamic pattern in milk oligosaccharide expression over the course of lactation in our study mothers (56). The dominant oligosaccharide in the first year remained 2'-FL (H-2), however, as a percentage of the total, it increased to about 14 weeks, at which time it began a gentle decline. LNF-I (H-1) as a percentage of total oligosaccharides declined from being the second-most common milk oligosaccharide in the first weeks postpartum (25% of total) to only about 10% of the total at the end of the first year, similar to several other oligosaccharides. In contrast, 3-fucosyllactose (Le^x) as a percentage of total oligosaccharide continues to increase consistently throughout the period shown. It is noteworthy that by the end of the year the contribution from percent H-2 and percent Le^x becomes very similar (approximately 30% of total oligosaccharide), while at one year postpartum the other oligosaccharides are found at very low level (approximately 10%). This longitudinal study indicates the importance of understanding the biologic relevance of the individual oligosaccharides and the complex interaction between the pathogen, host, and milk composition.

Erney et al analyzed milk samples from our cohort and from samples collected in a number of other countries and identified that the same milk oligosaccharides were found in all study populations, but with variation in the mean concentration of 2-linked fucosylated oligosaccharides between populations and between mothers within populations (55). All of the Mexican mothers were found to have 2-linked oligosaccharides in their milk, whereas about 20% of mothers of European descent lacked such structures (phenotypic nonsecretors).

Transgenic mice. As described under specific aim 2 above, we conducted a set of experiments which showed that mice litters born of transgenic dams who express α1,2 fucosyl glycoconjugates in their milk are protected against Campylobacter infection. Our data strongly support the role of α1,2 fucosyl glycoconjugates in the protection against Campylobacter infection and suggest that the main intestinal ligands for Campylobacter are the H-2 histo-blood group antigens.

Specific Aim 4. Develop strategies for large-scale synthesis of fucosylated glycoconjugates active against Campylobacter infection.

Over the past year, we have undertaken work and discussion with the Glycobiology Core (Dr. Newburg, with consultation from Dr. Seeberger) to develop strategies for synthesis of fucosylated oligosaccharides, the details of which are provided in the Methods section of the Stable Toxin (ST) project (specific aim 1). The three major strategies for the synthesis of oligosaccharides are: purely chemical techniques, enzymatic techniques, or synthesis by genetically altered organisms. Each has advantages and disadvantages when scaling up to large scale synthesis. Our first choice is chemical synthesis. We use two strategies for our chemical synthesis, the first being automated synthesis by the techniques in use in the laboratory of Dr. Seeberger, a collaborator to the Glycobiology Core. However, this technique has not yet been adapted to the synthesis of structures containing amino sugars. If a simple automated synthesis for any of the structures needed cannot be devised, these structures will be synthesized by classical organic carbohydrate synthesis by the Glycobiology Core, as described in the ST Project.

In the initial phases of this project, a combinatorial library of fucosylated oligosaccharides are being synthesized such as to include all possible Lewis epitopes found in human milk. Among the uses of this library is to measure their ability to inhibit Campylobacter and related pathogens. For this project the H-2-containing structure and its related compounds will be the first priority for testing. Ultimately all members of the library will be tested, individually and in combination, to ensure maximizing our ability to find the most active combination of epitopes. For the most active epitope or epitopes the synthesis will be scaled up and synthesized in kilogram quantities in a Good Manufacturing Practices (GMP) laboratory. These

oligosaccharides will then be tested in animals for their efficacy in inhibiting Campylobacter and related pathogens and for safety at doses that exceed their effective doses, ultimately leading to human testing.

If the active structure proves to be one that can be synthesized through genetically altered microorganisms, these techniques will be explored with the final purification and analysis taking place in a GMP laboratory.

Summary of our progress. Studies on the characterization of the *iam* locus show that the invasion process is complex, and may involve several genes, including those contained in this locus. The recently developed techniques will help us to phenotypically characterize the cohort mothers in relation to the histo-blood group antigens in human milk, relate the phenotype to maternal genotype, and thereby extend the studies on protection of breastfeeding children in the current cohort. The identification of a putative adhesin of Campylobacter may be important for its use as an immunogen to induce an acquired immune response against Campylobacter infection. One of the most important findings of the period is the demonstration of protection conferred by H-2 glycoconjugates, which was seen in children protected by H-2 epitopes contained in human milk, and protection of mouse pups whose transgenic dams produced milk containing H-2 epitopes.

Publications. See the Core section.

D. Research Design and Methods

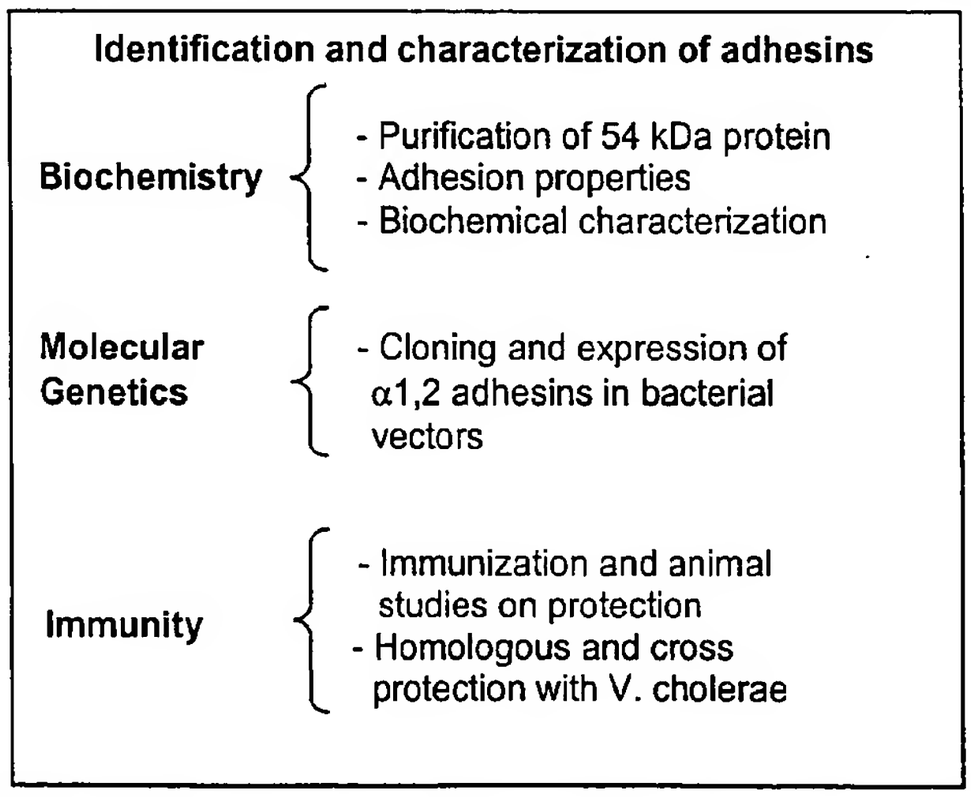
This section is divided in two major parts. The first part describes the experimental design and strategies that will be followed to address the specific aims. The second part deals with the methods that will be used to fulfill these aims.

RESEARCH DESIGN

Specific Aim 1. Characterize the Campylobacter adhesin associated with binding to host cell receptors.

Three strategies will be used to fulfill this aim. We will: 1) identify and characterize the 54-kDa agglutinin of *C. jejuni; 2)* using microarray technology, define the genetic differences between invasive and non-invasive strains; and 3) define the role of the protein glycosylation locus (*pgl*) in bacterial adherence to host cell and glycosyl receptors. Each of these strategies is described as follows:

1) Identification and characterization of the 54-kDa agglutinin of *C. jejuni* will be achieved as shown in the research schema to the right. Further purification will utilize two-dimension gels and blotting using hyperimmune serum against the partially purified 54-kDa agglutinin (54). A genomic library from a pathogenic *C. jejuni* strain will be constructed using the λgt11 expression vector. The expressed agglutinin will be screened using the hyperimmune serum. To further analyze the properties and sequence of this agglutinin-related insert, transformant cells will be subcloned in XL1-Blue *Escherichia coli* using plasmid pUC19 as a vector. The expression of this agglutinin in *E. coli* will be analyzed by blotting and by agglutination with HEp-2 cells and transfected *FUT1* CHO cells. Sequence analysis and homology will identify and characterize the gene or genes related to this agglutinin. To determine whether this protein could



be used as an immunogen to prevent Campylobacter infection, protection studies will be done by immunizing BALB/c mice orally and parenterally with the recombinant protein, followed by a challenge with Campylobacter. If colonization is prevented in immunized animals, passive protection studies will be performed by immunizing dams with the recombinant agglutinin and challenging the breastfed litters with Campylobacter. Cross-protection studies with *V. cholerae* will determine whether immunization of animals with a Campylobacter adhesin that binds to α1,2 fucosylated residues of gut epithelial cells also prevents *V. cholerae* infection (Specific Aim 2).

2) Genetic differences between invasive and non-invasive Campylobacter strains will be determined by microarray technology. To determine whether there are genetic differences between strains that may be responsible for the ability of Campylobacter to adhere to and invade gut mucosa epithelial cells, microarrays containing a representation of the complete genome sequence of prototype *C. jejuni* strain 11168 and the complete pgl will be used to study 6 different well characterized *C. jejuni* isolates, 3 pathogenic and 3 non-pathogenic. The pathogenic strains to be used in this study were isolated from children with diarrhea, have a high cell invasion index *in vitro*, show strong colonization in the mouse model, and possess the iam marker of invasion, but they have different RAPD fingerprinting. The non-pathogenic strains to be used in this study were isolated from asymptomatic children, are non-invasive *in vitro*, are poor colonizers in the mouse model, and have different RAPD fingerprintings; one possesses the iam marker while two do not. The genomic content of these strains will be determined using the *C. jejuni* whole genomic microarray, which contains 1360 unique Campylobacter genes and 13 additional genes of pgl. Reference probes of strain 11168 and probes from pgl will be used for construction of the microarray. Differences in gene content between tested strains will be analyzed using the Stanford Microarray Database (39), and by average hierarchal clustering using cluster software and displayed using TreeView.

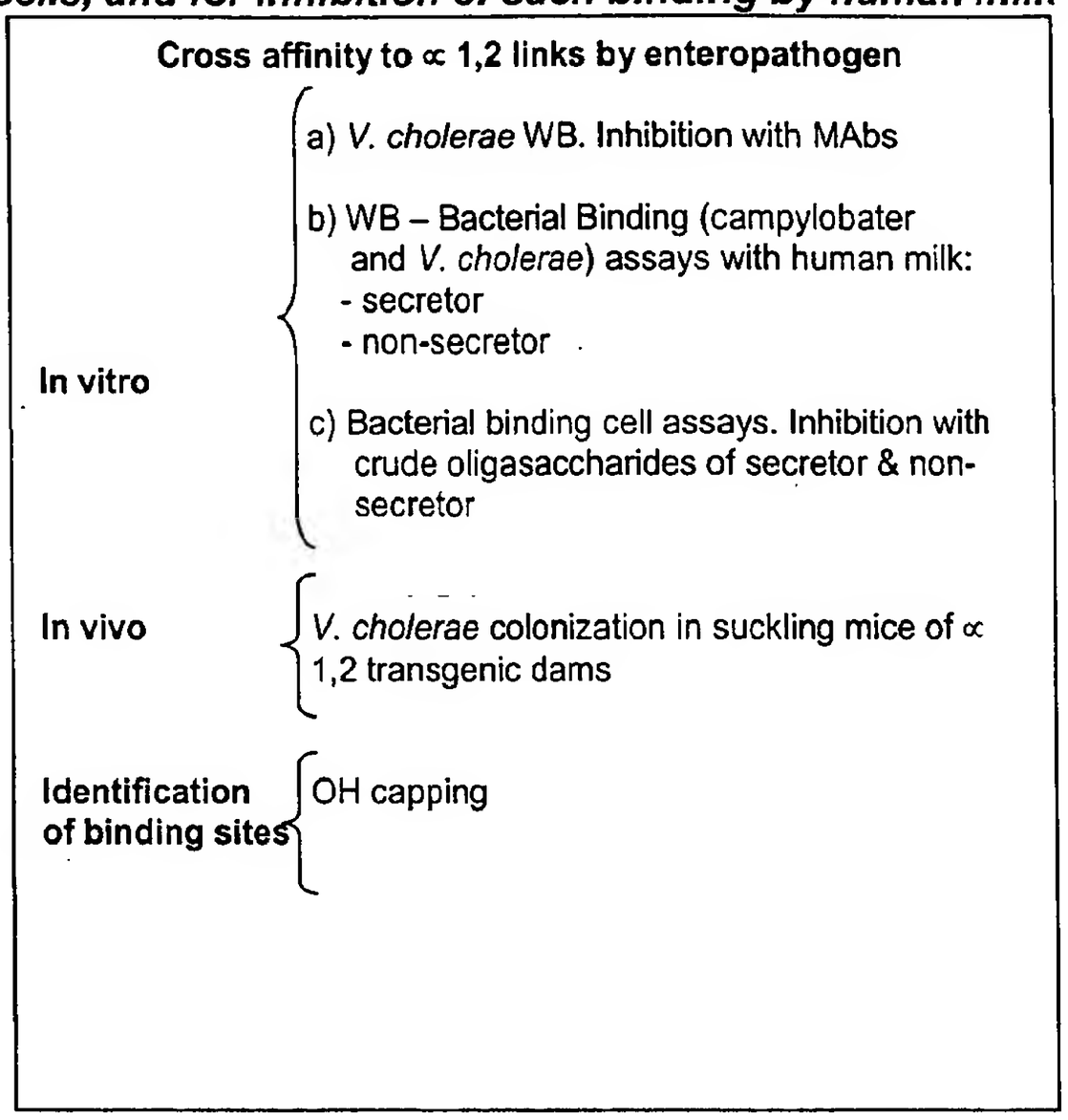
3) The role of the protein glycosylation locus (*pgl*) in bacterial adherence to host cell and glycosyl receptors will be determined. Carbohydrates play an important role in Campylobacter adherence. We will investigate whether this process depends on carbohydrate to carbohydrate interaction between Campylobacter surface glycoproteins (regulated by pgl) and glycosyl receptors of host cells, or on the interaction of Campylobacter adhesin proteins with glycosyl receptors of the host cell, plus the binding of lectin proteins expressed in host gut mucosa with the surface glycoproteins of Campylobacter. To explore both possibilities, we will construct insertional knockout mutants of a representative pathogenic strain in at least six different pgl genes (43-44). Mutants will be further analyzed for their ability to adhere to HEp-2 cells and *FUT1* transfected CHO cells.

Anticipated results and critique. There is no guarantee that immunizing mice with the 54-kDa agglutinin will protect them against Campylobacter colonization. However, there are data suggesting that it may be used as an immunogen to prevent colonization in immunized animals. Firstly, it is a surface protein that is released by deoxycholate, an important component of bile that has been seen to induce the expression of proteins involved in the adherence and invasion of Salmonella and Yersinia. Secondly, we have observed a serum immune response against the 54-kDa agglutinin in children infected with invasive Campylobacter.

We do not expect to have major problems on the implementation of the microarray techniques since this methodology has been extensively used in our laboratory for tuberculosis by Dr. Midori Kato, a collaborator on this project. One problem that could arise is that the genome sequence that will be used for hybridizations is that of reference *C. jejuni* strain 11168, known to be weakly adherent and lacking some genes that seem to be related with colonization and invasion (42). This problem could be overcome by adding to the hybridizing template sequences of such genes.

Specific Aim 2. Define the molecular basis for affinity of Campylobacter and V. cholerae to α1,2-linked histo-blo d group antigen of epithelial cells, and for inhibition of such binding by human milk oligosaccharid s.

In vitro studies have shown receptor-like glycocompounds in human milk that inhibit classical and El Tor *V. cholerae* cell adherence, and our preliminary data demonstrate the affinity of *V. cholerae* for neoglycoproteins of the tissue-blood group antigens immobilized on nitrocellulose membranes, similar to what is observed with Campylobacter. These data suggest that *V. cholerae* and other enteropathogens may share with Campylobacter the same mechanism of initial binding to gut mucosa. It is therefore important to elucidate the molecular basis of the binding of *V. cholerae* to epithelial cells and the role of fucosylated residues in this binding. We will apply the same models that we have used to study Campylobacter cell adherence (see research schema to the right).



Inhibition studies of *V. cholerae* binding to tissue-blood group antigens will be done by Western blot of neoglycoproteins immobilized on nitrocellulose membranes, utilizing monoclonal antibodies specific for Lewis ABH antigens to define binding specificity. To further characterize the role of milk oligosaccharides in *V. cholerae* binding, Western blots will be done by transferring and immobilizing glycocompounds of milk from secretor and non-secretor mothers, and milk from transgenic mice with the *FUT1* gene with the whey promoter expressed in mammary gland, using non-transgenic mice as controls.

Inhibition of *V. cholerae* binding to intestinal cells and CaCo-2 cells will be done with neutral and fucosylated milk oligosaccharides to determine the minimal concentration required to prevent binding. To further define the role of α1,2 linkages in bacterial cell binding, crude milk oligosaccharides from secretor and non-secretor mothers will be used at different concentrations in intestinal cell and CaCo-2 cell assays.

In vivo studies. To demonstrate that *V. cholerae* colonization is also inhibited by human milk α1,2 fucosyl glycoconjugates, as in Campylobacter, *in vivo* studies will be done by challenging suckling mice from α1,2 transgenic dams with *V.cholerae*. We will use B6/SJL mice transfected with a plasmid containing the human α1,2 fucosyltransferase gene (*FUT1*), with a polyA signal for bovine growth hormone, and a murine whey acidic protein promoter that directs expression primarily to lactating mammary gland (48). Preliminary studies on *V.cholerae* colonization in these mice showed a shorter colonization time of approximately 10-15 days. Thus, the *in vivo* studies with *V. cholerae* will be shorter than studies with Campylobacter, with experiments lasting only 10 days after challenge. There will be 4 groups of animals: control mice (group 1) will be inoculated only with saline (sham); groups 2 to 4 will be challenged with 10^2, 10^6, and 10^7 CFUs of El Tor strain, respectively. Colonization will be checked on days 1, 4, 7, and 10 in ileum and jejunum segments of sacrificed mice; *V. cholerae* will be identified by immunofluorescence and CFUs per cm^2 of gut.

Identification of binding sites. It has been suggested that the binding of the carbohydrate binding protein of *V.cholerae* to the H-2 tetrasaccharide is dependent upon the presence of calcium ions. In particular, the binding relies on the interaction of the C2- and C3 hydroxyl groups of the fucosyl moiety with calcium. To test this proposal, we will prepare H-2 analogs in which either one or both of the hydroxyl groups in question will be alkylated (Figure 14). Using mono- and dimethylated fucosyl donor 20, reaction with support-bound trisaccharide 7 will result in fully protected tetrasaccharide 21. Removal of the protecting groups using

standard conditions will reveal the desired methylated tetrasaccharides of the type 22. Alkylation is expected to prevent the interaction of the hydroxyl group with the calcium ion, and the specific contacts necessary for binding will be elucidated. The affinity of H-2 tetrasaccharide and H-2 analogs for *V.cholerae* and Campylobacter will be tested by inhibition assays of bacterial cell binding, using HEp-2 cells and *FUT1* transfected CHO cells. The Campylobacter assays will be done with a fluorescent transformed strain engineered in our laboratory using the pathogenic *C. jejuni* strain 287 that carries plasmid pJTR008.12 which replicates in this 287 strain and harbors the green fluorescent protein gene.

Anticipated results and critique. The experiments that will be used to define the affinity of *V.cholerae* to α1,2 linked histo-blood group antigens are the same that were used to determine the affinity of Campylobacter to these antigens; therefore, we do not expect to have any problems in this area. A potential problem could be the *in vivo* studies of colonization in suckling litters of α1,2*FUT* transgenic dams. In our preliminary studies we noticed that colonization with *V. cholerae* in wild B6/SJL mice was transient, lasting only 2 or 3 weeks. It is possible that the dams'-milk containing α1,2 fucosyl glycoconjugates do not clear colonization significantly faster than for the time needed for clearance to occur naturally. In this case, a new transgenic breed of mice, such as BALB/c mice, will have to be developed.

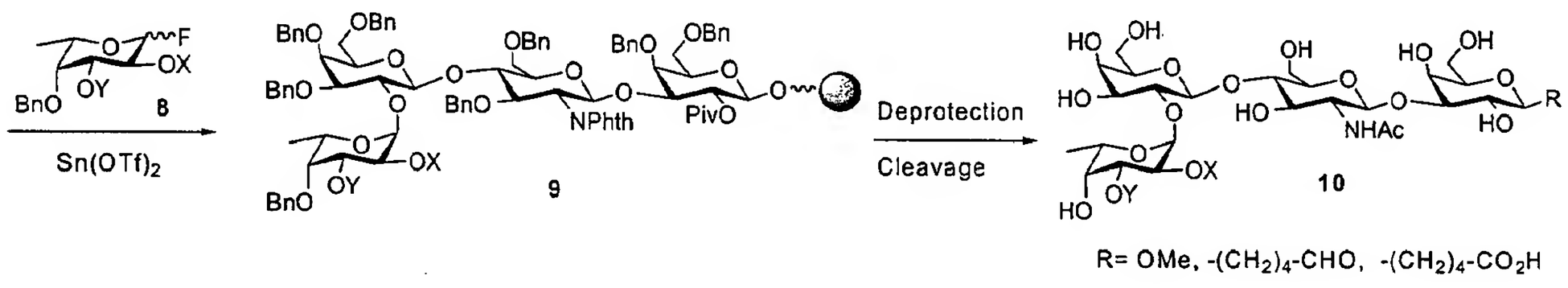


Figure 14: *Synthesis of an H-2 analog containing a "capped" fucose unit.*

Specific Aim 3. Determine the genetic polymorphisms that underlie maternal and infant secretor and Lewis phenotypes, and relate these to heterogeneity in human milk oligosaccharide expression and risk of bacterial diarrhea in the breastfed infant.

To define differences in susceptibility to Campylobacter and other bacterial enteropathogens (enteropathogenic [EPEC] and enterotoxigenic [ETEC] *E. coli*) according to secretor status, it will be necessary first to determine the secretor phenotype and genotype in the Mexican study population (see research schema on the next page). Based on our preliminary data, non-secretors are rare in the Mexican population; we estimate this frequency at approximately 2%. However, our phenotypic data and published literature describing non-European populatons suggest that our study population may have up to 20% of the combined group of non-secretors and low or partial secretors. We estimate in our study population that approximately 14% are partial secretors, as measured by the amount of α1,2 residues produced in milk. The homozygous genotype of mutation 428 in the *FUT2* gene, which totally blocks the fucose transferring activity by a nonsense mutation in the se allele, is frequently found among Caucasians (20%) but, as described in our progress report, is rare in our Mexican cohort mothers (found in 2 of 139 [1.4%] women; of the 3 non-secretors identified by *U. europaeus*, 2 shared this mutation). It is possible that partial secretors and the remaining non-secretors are homozygous for mutations in other sites of the *FUT2* gene. Partial secretors are thus able to produce slight fucose transferring activity of the *FUT2* enzyme and therefore to synthesize small amounts of the H-2 type structures in milk, gut mucosa, and other secretions. A *FUT2* gene mutation in nucleotide 385 has been associated with this partial secretor status in East Asian population (50-52), and therefore it is possible that this or similar missense mutations are present in our cohort.

a) Identification of the secretor status and Lewis phenotype in the 415 Mexican mother-infant pair "genotype" cohort (see Core, Research and Design, figure 4). We will first determine whether cohort mothers are secretors, non-secretors, or partial secretors by Western blot of milk samples, and by ELISA we will measure total concentration of H type structures. In children, secretor status

will be determined by Western blot in saliva. Lewis phenotyping will be done in milk by using specific monoclonal antibodies to Le^a, Le^b, H-1, and H-2.

b) Determination of Se and Le genotype. Genotyping of *FUT2* (Se gene) and *FUT3* (Lewis gene) will be done in milk from mothers and in buccal cells in children, by a multiplex PCR -RFLP. For *FUT2* polymorphism, we will investigate the following mutations: A385T, G428A, C571T, C628T, 685delTGG, and G849A. For *FUT3*, mutations: T59G, T1067A, G508A, T202C, and C314T. Genotyping will be done first in non-secretors and partial secretors, as determined by Western blot and ELISA, hoping to identify in these groups homozygous to any of these mutations. It is possible that in some of the non-secretors or partial secretors we will not find these mutations; then, the whole Se and Le genes of these individuals will be sequenced to identify possible new mutations, and in this case studies on transferase activity will be done to confirm that there is indeed a lack of fucose transferring activity due to a missense mutation of these genes.

Genetic variability of histo-blood group antigens and susceptibility to bacterial enteric infections

Secretor status	Identification of secretor status: - Dot EIA: milk and saliva - WB: milk Quantification of ∝1,2 fucosyl glycoconjugates in milk.		
Histo-blood group phenotyping	Milk (H1, H2, Le^a Le^b) Saliva in children		
Se and Le genotyping	Mutations		
	FUT2:	A385T G428A C571T	C628T 685delTGG G849A
	FUT 3:	T59G T1067A G508A	T202C C314T

c) Definition of genotype subgroups. The analysis to evaluate differences in susceptibility to infection will be done based on the combination of genotypes, and three groups will be formed for each glycosyltransferase gene: wild type for Se/Se or Le/Le; heterozygous, Se/se or Le/le; homozygous, se/se or le/le. For the population under study, any of the missense or nonsense mutations will be considered as se/se. Therefore, we expect to identify among the 415 mother-infant pairs 16% of se/se (~60 mothers and ~60 children), most of which are expected to be partial secretors (~52 mothers and ~52 children), and a few of them (~8 mothers and ~8 children) with a 428 nonsense mutation (true non-secretors) (See Core).

d) Association between Se and Le genotype subgroups and Campylobacter and other enteric bacterial infections. To determine if different susceptibility to Campylobacter and other enteric bacterial infection is dependent on the Se and Le genotypes, the incidence of symptomatic and asymptomatic infections will be calculated in breastfed and non-breastfed children according to the genotype subgroups. First, we will describe, among the nine (or more, depending on gene mutations) distinct secretor and Lewis histo-blood group genotypes, the pattern in the mean maternal milk concentrations of 2-linked fucosylated oligosaccharides, particularly 2'-FL (H-2). For this specific aim we will utilize both current and previous cohorts (N=415). For hypothesis testing purposes, we will classify these genotypes into 3 genotype groups based on our current understanding of the potential dominance of the Lewis genes in relation to the secretor gene. Using the mean concentration of 2'-FL from a previous study, the available sample size of 415, α=0.05 and 2-tailed test, will provide > 90% power to detect significant differences among these three genotype groups, and >80% power to detect significant differences between any 2 groups. We also want to investigate the risk of Campylobacter infection among the genotype groups based on the infant saliva. Assuming that the incidence of invasive strains of Campylobacter infection is 0.11, 0.18 and 0.29, for non and low secretor, medium and high protection genotype groups respectively, we will have >80% power (given ∀=0.05 and a 2-tailed test) to detect a significant difference among these groups. This sample size will also provide us with 80% power to detect a 2.6-fold difference in the incidence of invasive Campylobacter strain between the low and high secretor genotype groups. For breastfed children,

the genotype status of the mother will be the independent variable, and the outcomes to be analyzed will be the incidence of overall diarrhea, Campylobacter diarrhea, and asymptomatic Campylobacter infection. Variables to be controlled as possible confounding factors will be age, gender, Campylobacter antibodies in milk, and genotype of the child. For the analysis of susceptibility of non-breastfed children, incidence rates of overall diarrhea, Campylobacter diarrhea, and asymptomatic Campylobacter infection will be analyzed according to the genotype status of non-breastfed children. Possible confounding variables to be controlled are gender and previous Campylobacter infection while breastfeeding. Similar analyses will be done with diarrheagenic *E.* coli and human calicivirus-associated diarrhea as described in the core and projects (see Core Aim 2).

e) Protective role of 2'-fucosyllactose (2'-FL) oligosaccharides in human milk against specific strains of Campylobacter and other causes of bacterial diarrhea. For this purpose we will use only the current cohort (n=306) because in this cohort, the laboratory in Mexico is typing the strains of Campylobacter. Thus, we can restrict analysis to invasive strains of Campylobacter, which is approximately 70% of the episodes of Campylobacter diarrhea in children (38). In the current cohort, the incidence of Campylobacter diarrhea is 0.26 cases per child-year of breastfeeding. In a recently submitted manuscript, we demonstrated human milk 2'-FL protection against moderate-to-severe diarrhea of all causes and Campylobacter diarrhea specifically, among the 93 breastfeeding mother-infant pairs in the previous cohort whose samples were analyzed. We will extend this research in the proposed grant cycle. Assuming an incidence rate of 0.18 for invasive strains of Campylobacter infections/child-year, the available sample size (n=306) will provide >80% power, given $\forall$=0.05 and a 2-tailed test, to detect a 2-fold protection by high vs. low levels of 2-linked fucosylated oligosaccharide and in particular 2'-FL (H-2) concentration or percent of total oligosaccharides in maternal milk. Further, we can examine 2'-FL in maternal milk as a time-varying factor in relation to protection against symptomatic, strain-specific Campylobacter infection. This project will also examine the association between the maternal milk 2'-FL phenotype and the concentration of anti-Campylobacter antibody in human milk (see Core Aim 1).

Anticipated results and critique. Based on the characteristics of the Mexican population, we expect a greater genetic diversity due to racial mixing between Amerindians and Europeans. Therefore, in addition to the genotype found among non-secretor Europeans, it is very likely that we will find one or more different non-secretor genotypes, unique for this population. In our collaboration with the Molecular Biology Core, we can access the expertise of Dr. Jacques LePendu, who is recognized in histo-blood group antigen research, who will, along with Dr. Jiang, help guide the genotyping studies.

Specific Aim 4. Measure the safety, tolerance, and efficacy of the human milk oligosaccharide 2'-fucosyllactose and related compounds from human milk against Campylobacter and related pathogens in mice.

We propose preclinical trials to determine the toxicity of 2'-FL and 2'-FLNAc at concentrations several times higher than those found in human milk, to determine the adequate interval of daily intake, and to clarify their usefulness as prophylactic and/or therapeutic substances.

Safety and tolerance. Three-week-old BALB/c mice will be fed during 7 days either BID (twice daily) or TID (thrice daily) with escalating doses of 2'-FL or 2'-FLNAc starting at 2 mg per intake (normal average concentration in human milk) up to 200 mg. Animals will be followed for 2 weeks after the last dose to evaluate food tolerance, weight, presence of diarrhea, and abnormal behavior (Table 1).

Table 1. Short term safety and tolerance studies in 3-week-old BALB/c mice.

Group	No. Mice*	Dose per intake	2'-FL or 2'FLNAc BID (mg) Dose per day	2'-FL or 2'FLNAc BID (mg) Total dose (7 d)	2'-FL or 2'FLNAc TID (mg) Dose per day	2'-FL or 2'FLNAc TID (mg) Total dose (7 d)
1	10	2	4	28	6	42
2	10	10	20	140	30	210
3	10	20	40	280	60	420
4	10	100	200	1400	300	2100
5	10	200	400	2800	600	4200
Control	20	Saline (control)				
Total	70*			23,240		34,860

*Per experiment. The total for all experiments=50 test group (group A and B) mice X2 test oligosaccharides X2 treatments (BID, TID)=200 test mice. Plus 20 control mice=220 total.

Prophylaxis studies. Our previous studies demonstrated that neutral oligosaccharides from human milk at concentrations equal to those found naturally and given 2 h before, during, and 2 h after challenge with Campylobacter significantly reduce intestinal colonization in BALB/c mice. In the present proposal we will extend these studies to evaluate different concentrations of more simple structures such as 2'-FL and 2'-FLacNAc, administered 2 days before challenge, during challenge, and 2 hours after challenge. If these carbohydrates are well tolerated, doses of 2, 10, 20, and 40 mg per intake will be given BID or TID. There will be 10 animals in each dose group plus one positive and one negative control group (see F. Vertebrate Animals section below). Animals will be challenged with 2 different concentrations of Campylobacter. Colonization will be assessed daily for 2 weeks by determination of CFUs per gram of feces.

Treatment studies. To define whether fucosylated oligosaccharides have a therapeutic effect in Campylobacter infected BALB/c mice, treatment will be given at escalating doses to determine the minimal therapeutic dose. Groups of 10 mice each will be formed and given escalating doses starting at 2, 10, 20, and 40 mg, per intake BID or TID for 7 days. This will be done to find the minimal dose that is well tolerated and clears Campylobacter colonization. Animals will be monitored for Campylobacter colonization by daily quantitative stool cultures during the 7 days of treatment and if Campylobacter is eradicated, animals will be followed for one month more with weekly stool cultures to confirm eradication. In addition to testing for treatment of campylobacter, we plan a parallel study of the treatment of *V. cholerae*, as described under the Vertebrate Animals section below.

Anticipated results and critique. It is possible that the frequency with which oligosaccharides will be given (BID and TID) will not be sufficient to clear completely Campylobacter colonization. In this case, it may be necessary to include another group of experiments in which the oligosaccharides are given mixed with drinking water to increase intake frequency.

METHODS

Milk fraction. Oligosaccharide fractions were obtained from pooled human milk as previously described (25). TLC of neutral and fucosylated fractions indicated that each fraction contained distinct oligosaccharide components. This will be performed in collaboration with the Glycobiology Core. All fractions will be tested for their ability to inhibit association of *V. cholerae* with intestinal cells and *FUT1*-transfected CHO cells.

Bacterial strains. Prototype invasive *C. jejuni* strains 166-IP and 287-IP from children with inflammatory diarrhea; *C. jejuni* strain 50-SP, from a healthy child; and two *V. cholerae* strains, El Tor and Classic, will be used for these studies.

Campylobact r/HEp-2 cell adherence assay. Campylobacter binding to a monolayer of HEp-2 cells will be performed as previously described. (26,27). Briefly, HEp-2 cells are grown to confluency and transferred into multi-chambered culture slides (Falcon, Franklin Lakes, NJ). For inhibition assays, 100-µL suspensions of 9×10^8 bacteria of each microorganism, previously incubated with the test milk fractions, are added to monolayers of HEp-2 cells in the 8-chamber tissue culture slides, incubated at 37 °C for 5 h, washed, and stained with Warthin-Starry. Results of triplicate assays are given as the percent inhibition of bacterial-cell association, relative to identical positive controls to which no milk fractions are added.

Bacterial binding Western blot. To assess the ability of Campylobacter and *V. cholerae* to bind to histo-blood group antigens, bacterial binding Western blot assays are performed with DIG-labeled bacteria (23,24). Neoglycoproteins of blood group antigens are applied to lanes for SDS-PAGE at 6.3×10^{-10} M oligosaccharide per lane. Membranes are washed in TBS, immersed in a DIG-labeled bacterial suspension of 0.2 OD600 and incubated 4 h at room temperature with gentle stirring. Membranes are then washed and incubated for 1 h with the alkaline phosphatase-conjugated anti-DIG antibody, washed and stained with X-Phosphate (5-bromo-4-chloro-3-indolyl phosphate) and Tris-buffered nitroblue tetrazolium in saline (pH 9.5) substrate (Boehringer Mannheim).

Specificity of *V. cholerae* binding to blood group antigens. This will be determined by transferring neoglycoproteins to nitrocellulose membranes, and preincubating with MAbs to H-1, Le^a, Le^x and Le^b antigens (Signet Laboratories) or H-2 and Le^y antigens (Accurate Chemicals) diluted 1:10 and 1:20. A 100-µL suspension of 9×10^8 CFU of DIG-labeled bacteria is added. Membranes are then incubated with anti-DIG alkaline phosphatase conjugate and stained with X-phosphate and nitroblue tetrazolium.

CHO cells transfected with human glycosyltransferases. α1,2 fucosyltransferase-transfected CHO cells (CHO-*FUT1*), α1,3/4 fucoslytransferase-transfected CHO cells (CHO-*FUT3*), and α1,3 fucosyltransferase-transfected CHO cells (CHO-*FUT4*), and parental CHO cells transfected with the vector $pCDM_7$ lacking the α1,2 *FUT* gene(CHO-WT) will be used to test bacterial binding and bacterial/host cell agglutination. Parental CHO cells with the vectors will be used as controls.

Bacterial binding to monolayers of transfected CHO cells. The binding of bacteria to CHO cells transfected with the human gene for α1,2-fucosyltransferase (*FUT1*), will be assessed by bacterial-cell association assay. Transfected CHO cells expressing the *FUT1* fucosyltransferase needed for the synthesis of human H-type antigen (α1,2 fucosyl residues) are grown to confluency (28). Controls are wild type CHO cells, parental CHO cells carrying only the plasmid vector, and a clone that expresses the murine UDP Gal:Galα1,4GlcNAcα1,3-transferase. Monolayers are harvested and seeded into each well of an 8-chamber slide and incubated for 18 h, washed and incubated with a suspension of 9×10^8 bacteria/mL. Wells are rinsed, fixed with 10% formalin for 1 h, stained by the Warthin-Starry method, and examined under oil immersion with light microscopy, or Confocal microscopy for mutant strains with the fluorescent plasmid. Identical preparations grown on round cover slips are examined by scanning electron microscopy after fixing in 2% glutaraldehyde, dehydration through a graded series of solvents, and surface gold deposition.

Inhibition of binding. α1,2 fucosyl ligands and homologs will be tested for their ability to inhibit binding of Campylobacter and *V. cholerae* strains to CHO-*FUT1* cells. For molecules that bind to H-2 ligands, including anti-H-2 monoclonal antibodies (anti-H-2 MAbs) and the lectins *Ulex europaeus* (UEA I) and *Lotus tetragonolobus* (Lotus), inhibition is measured on monolayers of CHO-*FUT1* cells incubated in 8-well chamber slides for 1h with each of the α1,2 fucosyl ligands before adding 100 µL of the bacterial suspension containing 1×10^8 bacteria/mL. For inhibition using homologs to cell surface receptors, including human milk neutral oligosaccharides (Neutral-OS), milk from secretor and non secretor mothers, neoglycoprotein BSA-H-2 (IsoSep AB, Tullingen, Sweden), and 2'fucosyllactose, 100 µL of the bacterial

suspension are incubated with each of the homologs before being added to the cell monolayer. In both assays, after a 3 h incubation at 37°C, wells are rinsed, lysed with 1% Triton X100, and CFU (colony forming units) of bacteria per well are determined. Data are interpreted as percent inhibition of bacteria association to cells relative to positive controls to which no α1,2 fucosyl ligands or homologs are added.

Cell agglutination. To measure the binding of bacteria to specific glycans, expressed on the cell surface of glycosyl transferase transfected CHO cells, Campylobacter and *V. cholerae* agglutination of a suspension of detached CHO cells is measured. Confluent monolayers of CHO-WT cells, CHO-*FUT1*, CHO-*FUT3*, and CHO-*FUT4* are detached with trypsin-EDTA. Aliquots (10 μL) of a $3x10^6$ cells/mL cell suspension are placed on microscope slides, and an equal volume of UEA I solution (1 mg/mL) or a suspension of 10^8 bacteria is added. Agglutination is determined in a blinded manner. Immediate agglutination is defined as 3+, an intermediate level of agglutination is 2+, perceptible but weak agglutination is 1+, and lack of agglutination is zero (0).

Mouse colonization. The effect of human milk oligosaccharides on *V. cholerae* colonization *in vivo* will be determined in BALB/c mice (weighing 10-20 g). All animal protocols have been reviewed and approved by the Animal Care Committee of the National Institute of Medical Sciences and Nutrition. Groups of 10 mice caged in pairs will receive orally oligosaccharides in 100 μL of PBS 2 h before, during, and after oral challenge with 10^4 or 10^8 CFU of *C. jejuni* 383-IP. Controls are given 100 μL of PBS before, during, and after challenge with the same bacterial suspensions. To test for any adverse effects due to the administration of oligosaccharides *per se*, another control group will receive only oligosaccharides. Shedding of Campylobacter will be determined by daily quantitative stool cultures for 7 days after challenge.

Expression of H-2 epitope in milk of transgenic mice. B6/SJL mice will be transfected with a plasmid containing the human α1,2 fucosyltransferase gene (*FUT1*), the poly A signal for bovine growth hormone, and a murine whey acidic protein promoter that directs expression primarily to lactating mammary gland. PCR of each dam will be used to verify that they are transgenic (48). Lactating female mice and litters will be caged individually, mice will be observed for three days, and stool cultures from mothers and cultured intestinal homogenates from one mouse of each litter will be used to ensure that they had not been previously infected. Suckling mice will then be orally inoculated with 10^2, 10^6 and 10^7 CFU cholera/animal and returned to their mother to continue nursing. Non-transgenic controls wil be pups of non-transgenic mothers; the pups will be inoculated with 10μL of 10^8 CFU/mL. To determine colonization, every third day for 10 days (day 1, 4, 7, 10) one mouse of each litter will be sacrificed. The data for each time point will consist of at least ten individual pups. 1 cm of the distal ileum and the cecum will be resected and placed separately in 1 mL of PBS, weighed, homogenized, and centrifuged at 2,000 rpm for 10 min. Serial dilutions will be cultured to determine CFUs per gram of tissue.

The measure of colonization will be expressed as the percentage of pups positive for cholera within each treatment at each time period tested. Differences between the pups nursing non-transgenic wild type dams and those nursing transgenic dams challenged with an inoculum of 10^6 cholera, will be evaluated by the Mann-Whitney test. Differences between all groups and across all times tested will be evaluated by analysis of variance followed by estimation of the regression coefficient.

EIA and Western-blot for quantification of H glycoconjugate in human milk. Total concentration of H glycoconjugate in human milk samples will be determined by a competitive enzyme-lectin assay and for a qualitative method, a Western blot will be used. For the immunoenzymatic method microplates are coated with *U. europaeus* lectin (1 μg/ml) and blocked overnight with Bovine serum albumin. For this competitive assay, Biotin-coupled H-2 at a concentration of 400 ηg/mL in 0.1% BSA/PBS is incubated with milk samples and with standard curve of H2-BSA, then washed and incubated with streptavidin-alkalin-phosphatase conjugate and developed with P-nitrophenylphosphate. A program of quality control is used for each run. Each plate is run with a standard curve, a negative, a positive, and a blank control. Coefficient of variation, and standard deviation are calculated for standard curves and controls. For immunoblots, milk samples are run in one-dimensional 12% sodium dodecyl sulphate polyacrylamide gel electrophoresis. Gels are blotted onto PVDF membranes. For subsequent lectin probing, blots are blocked in PBS-Tween and

incubated with peroxidase-coupled *U. europaeus* lectin at a concentration of 5 µmL in WBR-TBS, then developed with peroxidase substrate.

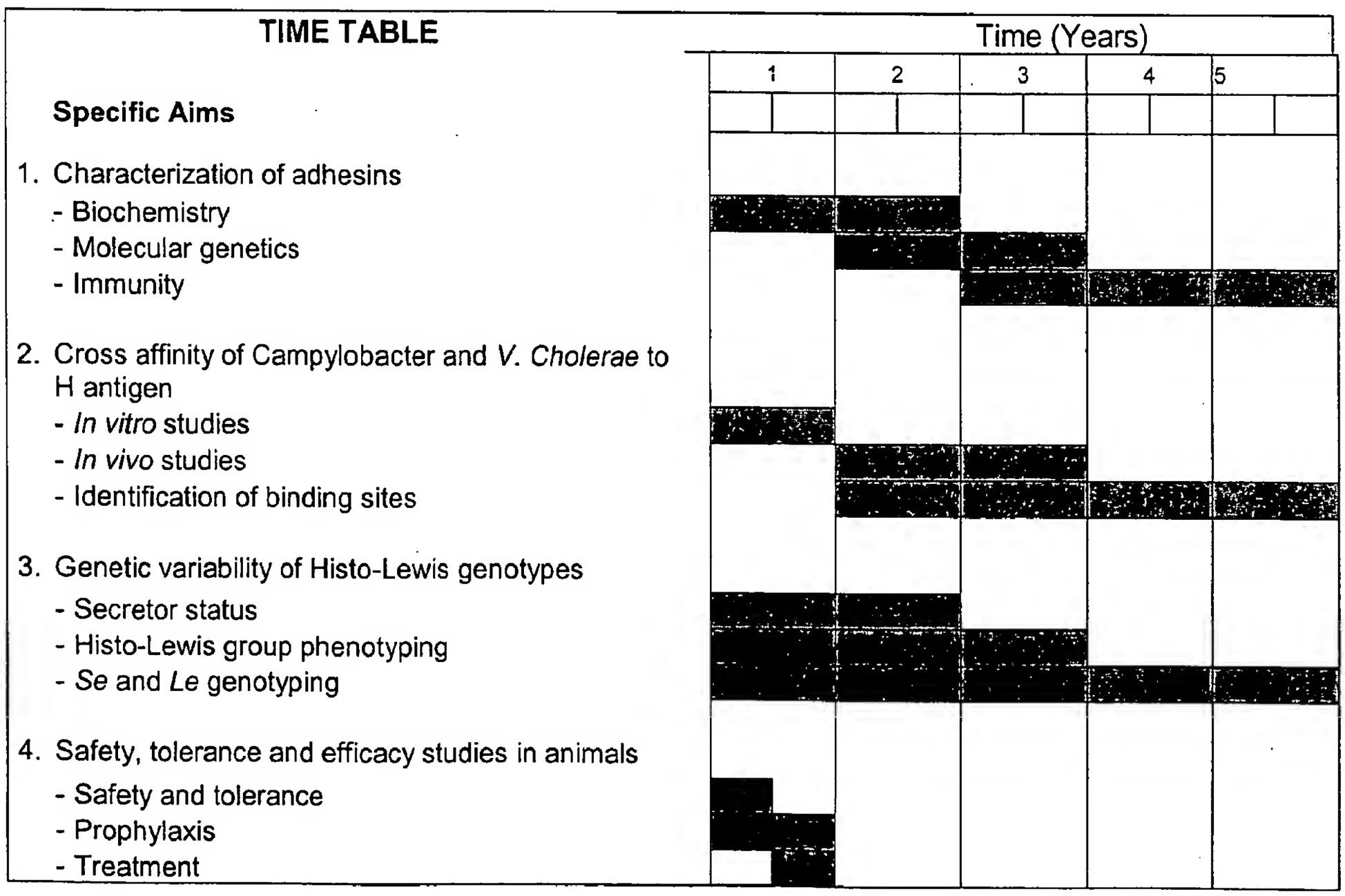
Construction of pJTR008.12, a green fluorescent plasmid. A shuttle plasmid containing the green fluorescent protein (*gfp* gene) of *Aequorea victoria* was constructed to transform invasive wild antibiotic multiresistant strains of *C.jejuni*. The plasmid was constructed by subcloning into the pJTR002 plasmid, the *gfp* gene with a consensus promoter of *C.jejuni* obtained from plasmid pMW1007, kindly provided by Dr. William Miller. Plasmid pJTR002, made in our laboratory, carries a *cat* gene of *C.coli* and is able to transform wild type invasive multiresistant Campylobacter strains, which the pMW1007 is not. The description of the construct is shown in the Figure 15 on the next page. This plasmid will be used to transform wild type invasive strains for studies to identify carbohydrate binding sites and studies on the affinity of synthetic carbohydrates (see Core).

Histo-Lewis genotyping by PCR. This method will be developed with the help of the Molecular Biology Core. The PCR methods that will be used for genotyping the secretor and Lewis genes have been previously described (49-51, 57). The entire *FUT* gene will be amplified using the following primers: F 5'-GCCTTTCTCCTTTCCCATGGCCCAC-3', and R 3'-CTTGATTACGACCGGGCAGGAAACT-5'. For the amplification of the 385 mutation, the following primers will be used. For the wild type 385: 5'-ACTGGATGAAGGAGGAATACCGCCACA-3'; and for the 385 mutation: 3'-AAGGGCCCCCTCATGCAGGCGAAGTGG-5'. restriction enzyme digestion will be performed with the following enzymes: *Ava*II for the 428 mutation; and *BstE*II, *Dde*I and *PinA*I for the 571, 628, 685, and 849 mutations. Gels are read in a GelDoc (BioRad, Cal. USA), and band polymorphism will be interpreted with a pre-established program on GelCompar (Applied Systems, Gent, Belgium).

Timeline

The following is our proposed timeline of work following the specific aims of our project. This work will be accomplished in collaboration with all cores and other project investigators.

TIME TABLE



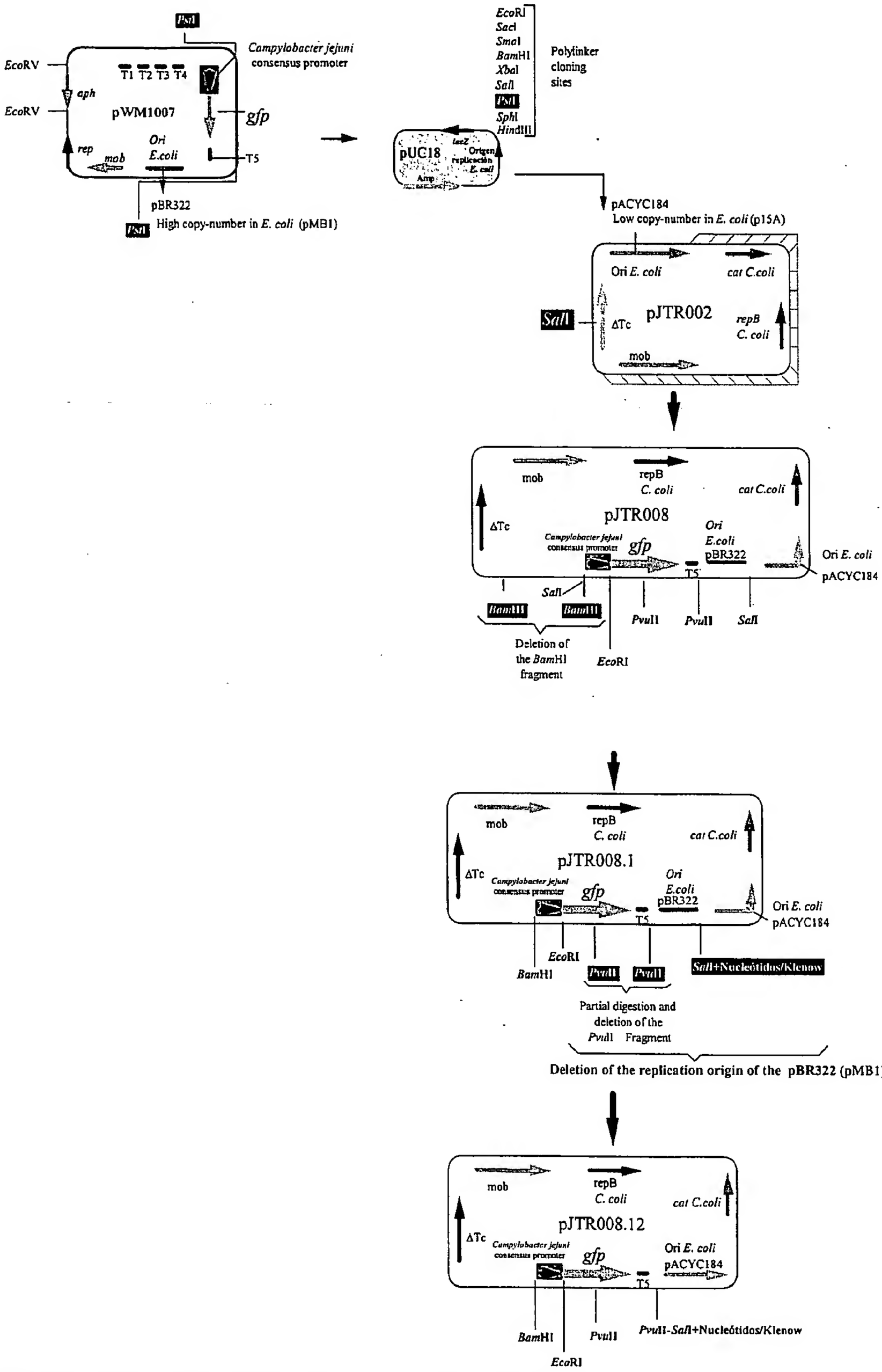


***Figure 15**. Construction of the fluorescent plasmid pJTR008.12.*

Abstract

Objectives: Human milk oligosaccharides containing α1,2-linked fucose inhibit several enteric pathogens from binding to host ligands. We tested the hypothesis that in breastfed infants the rates of diarrhea due to campylobacter, caliciviruses, and all causes are inversely associated with the quantity of 2-linked fucosylated oligosaccharides in maternal milk.

Study design: A cohort of 93 breast-feeding mother-infant pairs in Mexico was followed up to 2 years postpartum with feeding and illness data collected weekly. Maternal milk samples obtained 1-5 weeks postpartum were analyzed to determine their specific and total 2-linked oligosaccharide content, measured as concentration and percent of milk oligosaccharide. Data were analyzed by Poisson regression.

Results: The milk of all mothers contained 2-linked oligosaccharide (range: 0.8 to 16.2 nmol/L, 50 to 92 percent of milk oligosaccharide). The incidence or campylobacter diarrhea (n=31 cases) was significantly (P=0.004) higher in infants whose milk contained low levels of a specific 2-linked oligosaccharide, 2'-fucosyllactose (2'-FL), as a percent of milk oligosaccharide. The incidence of calicivirus diarrhea (n=16 cases) was significantly (P=0.012) higher in infants whose milk contained low levels of another specific 2-linked oligosaccharide, lacto–*N*-difucohexaose I (LDFH-I), as a percent of milk oligosaccharide. The incidence of moderate-to-severe diarrhea of all causes (n=77 cases) was significantly (P=0.001) higher in infants whose milk contained low levels of total 2-linked oligosaccharide as a percent of milk oligosaccharide.

Conclusions: This is the first study to demonstrate the clinical relevance of human milk oligosaccharide as a mechanism of breastfeeding protection against infectious diarrhea. These results suggest the potential to develop oligosaccharides as novel prophylactic agents to prevent diarrhea in susceptible populations.

Introduction

Breast-feeding provides significant protection against diarrhea in infancy (1-5). This protection, however, is not complete, and some breast-fed infants experience multiple episodes of moderate-to-severe diarrhea. Risk of diarrhea in breast-fed infants can be explained in part by lack of exclusive breast-feeding and environments that produce high dose exposure to enteric pathogens (1,5). Variation in the composition of protective factors in human milk could also account for variation in risk of diarrhea among breast-fed infants (5-9).

Human milk contains a variety of bioactive agents, including oligosaccharides, which are part of the innate, or genetically determined, defense system (5-10). Oligosaccharides are the third largest solid constituent of human milk after lactose and lipid (7-10). More than 130 distinct oligosaccharides have been characterized, but milk may contain more than 900 oligosaccharide structures ranging in complexity from 3 to 32 sugars (11). The most common human milk oligosaccharides are fucosylated and range from 3 to 8 sugars. The fucose may be connected to the oligosaccharide by an α1,2 linkage catalyzed by a transferase produced by the secretor gene (*FUT2*), or by an α1,3 or α1,4 linkage catalyzed by transferases produced by the Lewis gene family (*FUT3* and related genes). The fucosyltransferase genes involved in milk oligosaccharide synthesis also control expression of the Lewis blood group type (12-14). The expression of milk fucosyloligosaccharide varies significantly between mothers as a result of genetic polymorphisms (8,9).

Since 1987, investigators in the NICHD-sponsored Human Milk Program Project have examined adaptive and innate mechanisms of human milk protection against diarrheal diseases (4,6,8,9,15-28). In recent years, a major focus has been on the fucosylated oligosaccharides in relation to inhibition of specific enteric pathogens. Our *in vitro* and *in vivo* research has shown

that human milk oligosaccharides containing α1,2-linked fucose inhibit host cell binding to several enteropathogens (15,16,18-21), including *Campylobacter jejuni* (18,19) and caliciviruses (20,21). Campylobacter binding appears to be inhibited by a specific 2-linked fucosylated oligosaccharide, 2'-fucosyllactose (2'-FL). For many common strains of calicivirus, binding appears to be inhibited by several different 2-linked oligosaccharide structures. To examine the clinical relevance of the 2-linked oligosaccharides in maternal milk, we used a cohort study previously conducted in Mexico City as part of the Human Milk Program Project. The hypothesis of the present study is that in breastfed infants the rates of diarrhea due to *Campylobacter jejuni* and caliciviruses are inversely associated with the quantity of one or more of the major specific 2-linked fucosylated oligosaccharides found in maternal milk: lacto-*N*-fucopentaose-I (LNF-I), 2'-FL, lactodifucohexaose I (LDFH-I), and lactodifucotetraose (LDFT). Further, we speculated that the 2-linked human milk oligosaccharides in general might have population-level impact on prevention of diarrheal diseases in general. Thus, we also tested the hypothesis that the overall rates of diarrhea in the study population are inversely associated with the total quantity of 2-linked oligosaccharides in maternal milk.

Methods

Study design

The present study involves the analysis of data and banked samples from 93 mother-infant pairs who previously participated in a larger cohort study that was designed to examine breastfeeding protection against diarrheal diseases. Below we describe the original cohort study and the selection criteria and measures specific to the present study.

From March 1988 through December 1991, a cohort of 316 mother-infant pairs was enrolled and monitored from birth to two years postpartum in San Pedro Martir, a transitional neighborhood of Mexico City (6,22-25). Enrollment was restricted to term, normal birthweight infants. This research was approved by the institutional review boards of the Instituto Nacional de Ciencias Medicas y Nutricion in Mexico, the University of Texas Health Science Center at Houston, and Cincinnati Children's Hospital Medical Center. Written informed consent was obtained from the mothers who participated. Infant illness and feeding history were collected by trained field workers who made weekly home visits. Milk samples were collected from mothers weekly in the first month, and monthly thereafter. Samples were collected in the morning by an experienced study nurse using an Egnell electric breast pump to obtain the complete content of one breast. Samples were transported on ice from the study household to the laboratory, where they were stored at –70 °C. Infant stool samples were collected weekly with additional samples obtained whenever diarrhea occurred. Diarrhea samples were routinely tested for *Campylobacter jejuni*, diarrheagenic *E. coli*, *Shigella*, *Salmonella*, *Aeromonas*, and rotavirus, as detailed in previous publications (6,22-25). Calicivirus testing of stool samples was later performed by enzyme-linked immunosorbent assay (EIA) and PCR; a positive result by either test was considered calicivirus positive (26,27). Diarrhea episodes were defined as three or more watery stools within a 24-hour period or loose-to-watery bowel movements that exceeded the child's usual daily stool frequency by two or more stools as determined by a study physician. Using the severity scoring system of Ruuska and Vesikari (22,29), an episode of diarrhea was classified as moderate-to-severe if the score was $\geq$10. Classification of disease severity was blinded, based on the standardized history of each diarrhea episode that was recorded by a study physician independent of milk oligosaccharide analysis. Diarrhea was attributed to campylobacter or

calicivirus if the pathogen was detected in a stool sample collected during or within seven days of an episode of diarrhea. Diarrhea episodes associated with two or more pathogens were excluded from pathogen-specific analyses.

In 1997, technicians unaware of the study aims or subjects' history followed a protocol to select stored milk samples from potentially eligible mothers. Of the mothers in the original cohort study, criteria for inclusion in the present study were as follows. They had to have breastfed for at least two weeks to provide a minimum contribution of follow-up time to contribute to the analysis (n=276 [87%]); participated in a follow-up survey that included collection of a maternal blood sample to test for blood group type, which was performed in the clinical laboratory in Mexico (n=185 [58%]); and had at least one vial of milk in storage that contained 2 mL or more of milk collected between 1-5 weeks postpartum. A total of 93 mother-infant pairs met all of these criteria, which was the approximate limit of laboratory capacity for milk oligosaccharide analysis. If more than one milk sample was available per mother, a single sample was selected based on the date of collection closest to 30 days postpartum.

Milk samples were transported to Boston and analyzed as described previously (28). Milk oligosaccharides were isolated, perbenzoylated, and resolved by reversed-phase HPLC (C-8) with an acetonitrile/water gradient and detected at 229 nm. This chromatography system produces eight major peaks in human milk samples, which correspond to the most common oligosaccharides of human milk: four 2-linked fucosylated oligosaccharides (LNF-I, 2'-FL, LDFH-I, and LDFT); two fucosylated oligosaccharides that are not 2-linked (lacto-*N*-fucopentaose II [LNF-II] and 3-fucosyllactose [3-FL]); and their two precursors (lacto-*N*-tetraose [LNT] and lacto-*N*-neotetraose [LNneoT]). Detection of these oligosaccharides in human milk samples was not adversely affected by cryogenic storage or freeze-thaw.

Statistical Analysis

Incidence rates of diarrhea were calculated as the total number of cases per 100 breast-feeding child-months, i.e., from birth to the end of breast-feeding or termination from study, whichever occurred sooner. A secondary analysis was conducted of the incidence of diarrhea during post-breastfeeding child-months, i.e., from the end of breastfeeding to termination from study. Time during diarrheal illness was not included in the denominator used to calculate incidence rates. Study outcomes were defined as the rates of diarrhea associated with *C. jejuni*, calicivirus, all causes of diarrhea, and all causes of moderate-to-severe diarrhea. The major independent variables were the specific and total 2-linked oligosaccharides characterized in terms of concentration (nmol/L) in milk and the percent of milk oligosaccharide (the quantity of specific or total 2-linked oligosaccharide divided by the sum of eight milk oligosaccharides measured in milk). The percent of milk oligosaccharide measure was used to correct for variability in oligosaccharide concentrations due to lactation physiology and/or sampling, collection, storage, and testing.

Correlations were analyzed among oligosaccharide measures. The associations between milk oligosaccharide measures and rates of diarrhea outcomes were examined using a generalized linear model with a Poisson link function. This model was selected as optimal for analysis of incidence rates with one or more outcomes per person, accounting for variable lengths of follow-up time. Potential interactions or confounding by maternal age, sociodemographic factors, degree of breastfeeding, and maternal Lewis and ABO blood group types (factors shown in table 1) were analyzed in relation to milk oligosaccharide measures and rates of infant diarrhea. In addition to milk oligosaccharide measures, significant ($p<0.01$) risk factors for infant diarrhea identified from univariate analyses were: infant birth order, maternal

age, percent breastfed, and maternal ABO group. These factors were included in multiple regression models but did not confound the associations between milk oligosaccharide measures and diarrhea outcomes, and were therefore not included in final regression models. Due to lack of confounding, final models included only specific or total 2-linked oligosaccharide expressed as a percent of milk oligosaccharide in relation to diarrhea outcomes.

Results

Study Population

The 93 mother-infant pairs in this study were monitored for 857 breast-feeding infant-months and 765 post-breast-feeding infant-months between birth and 2 years of age. None practiced exclusive breast-feeding. The study population (table 1) was comparable to the entire cohort (22-25) regarding risk of infant diarrhea and all sociodemographic, hygiene, and infant-feeding factors, except that mothers included in this study breast-fed longer (median duration 9 vs 5 months, $P<0.01$) and were more likely to complete a secondary education or higher ($P<0.01$) than those not included. These factors were not associated with oligosaccharide concentrations or percent of milk oligosaccharide and were thus unlikely to affect the internal validity of this study. Two-thirds of mothers were O blood group; nearly three-quarters were Lewis a-b+. The serologic classification for two mothers was Lewis a+b-, a classification considered to indicate obligate nonsecretors. However, since the milk of these two mothers contained α1,2-linked oligosaccharide, a finding inconsistent with being a nonsecretor, the discrepancy between milk and blood group phenotypes resulted in classifying the blood group type as indeterminate (Table 1).

Oligosaccharide Profile

2-Linked fucosyloligosaccharide was found in the milk of all 93 mothers (table 2). The concentration of fucosylated oligosaccharides was nearly evenly distributed between types 1 and 2 (Table 2), and comprised 73% of total milk oligosaccharide. The most commonly occurring oligosaccharides were 2-linked: 2'-FL (34% of total), LNF-I (25% of total), followed by LDFH-I, and LDFT. Lewis a-b- mothers had a significantly ($P \leq 0.01$) higher percent of milk oligosaccharide containing only a 2-linked fucose compared to Lewis a-b+ mothers (LNF-I, 30% vs 23%; 2'-FL, 42% vs 31%). Specific and total 2-linked fucosyloligosaccharides, whether expressed as concentrations in milk or as percent of milk oligosaccharide, were not associated with maternal sociodemographic factors or ABO blood group. Correlations between specific fucosyloligosaccharides expressed as milk concentrations ranged from r=-0.1 to +0.8, and as percent of milk fucosyloligosaccharide ranged from r=-0.5 to +0.6.

Protection against Diarrhea

A total of 234 diarrhea episodes were identified during breastfeeding (median of 2 diarrhea episodes per child; range, 0-12 episodes per child). The incidence of all diarrhea was 28.8 cases per 100 child-months of breastfeeding. A total of 77 (33%) diarrhea episodes were moderate-to-severe; the incidence of moderate-to-severe diarrhea during breast-feeding was 9.5 cases per 100 child-months. Among all diarrhea episodes, 40 were associated with *C. jejuni*, 25 with calicivirus, 10 with enteropathogenic *E. coli*, 9 with rotavirus, 5 with shigella, and 4 with stable toxin-associated *E. coli*. Excluding diarrhea episodes with detected co-infections, there were 31 diarrhea episodes associated with *C. jejuni* in 22 children, and 16 episodes of diarrhea associated with calicivirus in 13 children (Table 3). Twelve of 31 (39%) *C. jejuni* diarrhea episodes and 5 of 16 (31%) calicivirus diarrhea episodes were moderate-to-severe.

Rates of *C. jejuni* diarrhea were inversely (P=0.004) associated with 2'-FL as a percent of milk oligosaccharide (Table 3). Although LDFH-I as a percent of milk oligosaccharide was significantly (P=0.047) associated with risk, when entered into regression models with 2'-FL, did not persist as a significant risk factor for *C. jejuni* diarrhea. 2'-FL as a percent of milk oligosaccharide remained significantly inversely associated with rates of *C. jejuni* diarrhea, whether in univariate or multivariate models. For calicivirus diarrhea, several milk oligosaccharides tended towards protective associations (Table 3), but only LDFH-I as percent of milk oligosaccharide was significant (P=0.012). The dose-response relationships between specific milk oligosaccharides and incidence rates of *C. jejuni* diarrhea and calicivirus diarrhea are illustrated in figure 1 (Panels A and B),

We found a significant inverse association between the quantity of total 2-linked milk fucosyloligosaccharide measured as a percent of milk oligosaccharide and the rates of all moderate-to-severe diarrhea (n=77 cases, relative risk [RR]=0.02, P=.001), but not all diarrhea (n=234 cases) in our predominantly secretor population. The inverse association between total 2-linked milk oligosaccharide and moderate-to-severe diarrhea was found to be a linear dose-response relationship (Figure 1, Panel C) when analyzed categorically (high, medium and low levels of total 2-linked oligosaccharides as a percent of milk oligosaccharide, n=31 per group). The inverse association between total 2-linked milk oligosaccharide and rates of moderate-to-severe diarrhea persisted throughout the breastfeeding period, but not in the post-breastfeeding period. Analysis of milk oligosaccharide that did not contain 2-linked fucose indicated that increased 3-FL as a percent of milk oligosaccharide was associated with increased rates of moderate-to-severe diarrhea (P<0.001) in breastfed infants. It may be that 3-FL serves as a biomarker for lack of 2-linked oligosaccharides in human milk.

Discussion

In this study of breastfed Mexican infants, we found that low levels of specific 2-linked fucosylated oligosaccharides in their mother's milk was significantly associated with increased rates of pathogen-specific diarrhea. *C. jejuni* and caliciviruses, excluding co-infections, together accounted for 20% of all diarrhea episodes in our study population. A low level of maternal milk 2'-FL as a percent of milk oligosaccharide was associated with a high rate of *C. jejuni* diarrhea in breastfed infants. Similarly, lower levels of maternal milk LDFH-I as a percent of milk oligosaccharide showed a dose-dependent association with higher rates of calicivirus diarrhea. We also found a broad, population health impact. Lower levels of total 2-linked oligosaccharide as a percent of milk oligosaccharide had a dose-response association with higher overall rates of moderate-to-severe diarrhea in the study population. The association between milk oligosaccharide measured during the first month postpartum and diarrhea in breastfed infants persisted through the course of breastfeeding, but did not persist after termination of breastfeeding. This observation was consistent with our proposed mechanism of protection: the presence of milk oligosaccharide in the infant gastrointestinal tract when exposed to pathogens. In our study population, all maternal milk contained some 2-linked fucosylated oligosaccharide. Nevertheless, genetic polymorphism resulted in a wide range of expression, with specific and total 2-linked fucosyloligosaccharide measured on a continuous scale. No association with rates of diarrhea in breastfed infants was observed when oligosaccharides were expressed as concentrations in milk, suggesting the importance of a denominator to correct for variability in milk oligosaccharide recovery that occurred despite standardized sample collection, storage, and laboratory methods.

This study addresses a fundamental mechanism of innate protection against infectious disease relevant to a variety of pathogens. The human milk fucosyloligosaccharides measured in this study are Lewis epitopes, products of the same genes that control maternal Lewis histo-blood group type. Blood group types are the result of genetic polymorphisms that determine oligosaccharide-containing glycoconjugate expression on host cell surfaces. Associations have been previously reported between histo-blood group type and differing susceptibility to bacterial and viral diseases. Glass et al. showed that O blood group individuals have increased susceptibility to cholera (20). P blood group type has been associated with susceptibility to hemolytic uremic syndrome (15). Ikehara et al. found an association between Lewis and secretor histo-blood group genotypes and risk of infection with *Helicobacter pylori* (16). Hutson et al. reported that O blood group individuals have increased susceptibility to Norwalk virus (36). Influenza virus binding has been shown to vary in relation to host blood group antigens (ref Boat et al). Raza et al found that secretor children have increased susceptibility to hospitalization for respiratory infections due to influenza viruses A and B, rhinoviruses, respiratory syncytial virus, and echoviruseses (ref).

Our study is unique in examining the mother-infant dyad. Our data indicate that at least in a predominantly secretor population, phenotypic variation in the relative quantities of 2-linked fucosyloligosaccharides in mothers' milk determines the protection offered to breast-fed infants. Because the same genotype that produces milk oligosaccharides in the mother is expected to produce cell surface receptors that increase risk in the infant, the lack of control for infant susceptibility is likely to have biased our results such that the true association between milk oligosaccharides and protection against disease is stronger than observed. Further, we note that certain major endemic pathogens not included in this study, e.g., ST-associated *E. coli* (14,21),

are also inhibited by 2-linked fucosylated oligosaccharides, while others, e.g., rotavirus, are inhibited by human milk glycoconjugates not known to be encoded by products of the secretor and Lewis genes (6). Thus, the association we have described provides only a glimpse into the potential protective role of the innate immune system of human milk.

A growing body of research suggests that common mechanisms of pathogenesis may exist between some bacterial and viral pathogens (**15-25,35,36**). We have found that both *C. jejuni*, a bacterium, and caliciviruses bind to 2-linked fucosyloligosaccharides (**22-25**). Fucosylated oligosaccharide milk fractions inhibit *C. jejuni* adherence to human epithelial cells *in vitro* and colonization in experimental mice, and 2'-FL inhibits *C. jejuni* binding to human intestinal mucosa *ex vivo* (**22,23**). Further, we have shown that Chinese hamster ovary cells transfected with a human fucosyltransferase gene bind *C. jejuni*, and that this binding is inhibited by Lewis epitopes containing 2'-FL (19). Our studies with caliciviruses have shown that Norwalk virus-like particles bind to tissue sections of the gastro-duodenal junction from secretors but not from nonsecretors (20), and that binding is blocked by milk from a secretor (16). Volunteers challenged with Norwalk virus become symptomatically infected only if they are secretors. Our data suggest that several different 2-linked milk oligosaccharide structures may inhibit binding by the most common strains of caliciviruses. We are continuing to characterize protection against specific strains of caliciviruses by specific milk oligosaccharides.

Research is needed to determine whether the simple milk oligosaccharide structures associated with protection in this study are themselves protective, whether they are biomarkers for more complex structures, or whether the range of simple to complex structures corresponds to degrees of binding affinity and protection against specific pathogens. Our findings indicate that heterogeneous expression of oligosaccharides in human milk provides infants with varying

degrees of protection against specific pathogens. Our findings are consistent with the concept that heterogeneous expression of oligosaccharides and their related glycoconjugates in the infant underlies individual susceptibility to different pathogens. Because many of the oligosaccharides found in human milk are unique, this study provides additional evidence of the importance of breast-feeding. In summary, this study provides novel clinical evidence that human milk oligosaccharides comprise a form of innate immunity for the nursing infant. Oligosaccharides may form the basis for oral agents with potent antibacterial and antiviral activities against emerging diseases.

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Table 1. **Characteristics of the study population: 93 breastfeeding mother-infant pairs**

Characteristic		Measure	Value
Number of people in the household		Median (range)	5 (3-11)
Animals kept in the household		No. (%)	66 (71%)
Age of mother (years)		Median (range)	23 (15-41)
Maternal education:	None/Elementary	No. (%)	42 (45%)
	Middle school		31 (33%)
	High school and beyond		20 (22%)
Primiparous mother		No. (%)	31 (33%)
Duration of breast-feeding (months)		Median (range)	9 (0.7-24)
Percent of infant feedings that were breast milk		Median (range)	49 (4-82)
Maternal Lewis blood group:	a-b+	No. (%)	67 (72%)
	a-b-		24 (26%)
	indeterminate		2 (2%)
Maternal ABO blood group:	O	No. (%)	62 (67%)
	A		18 (19%)
	B		12 (13%)
	AB		1 (1%)
Male infant		No. (%)	43 (46%)

Milk Oligosaccharide			Concentration (nmol/L)	Percent of total oligosaccharide
lacto-*N*-fucopentaose I		mean ± SD	3.21 ± 1.81	25.1 ± 10.3
		range	0, 7.0	0, 43.7
2'-fucosyllactose		mean ± SD	3.85 ± 1.04	33.7 ± 10.6
		range	0.5, 6.2	0.05, 66.7
lacto-*N*-difucohexaose I		mean ± SD	1.26 ± 1.04	9.5 ± 5.8
		range	0, 5.2	0, 25.4
lactodifucotetraose		mean ± SD	0.70 ± 0.75	5.1 ± 3.6
		range	0.03, 5.0	0.01, 16.9
Total 2-linked	OS	mean ± SD	8.37 ± 3.00	73.3 ± 9.1
oligosaccharide		range	0.76, 16.2	50.4, 92.3
Total milk	OS	mean ± SD	11.79 ± 4.5	100
oligosaccharide+		range	1.0, 31.5	

*Percent of oligosaccharide is the quantity of each specific oligosaccharide divided by the total quantity of the milk oligosaccharides/measured in this study.

+ Total milk oligosaccharide includes eight oligosaccharides: the four 2-linked fucosylated oligosaccharides, two non-2-linked oligosaccharides (3-FL and LNF II) and their two precursors.

Table 3. **Univariate analyses of specific α1,2-linked fucosyloligosaccharides in maternal milk and relative protection against *C. jejuni* and calicivirus-associated diarrhea in study children, by Poisson regression**

Milk	Campylobacter[†]		Caliciviruses[†]	
Oligosaccharide	β (SE)	P	β (SE)	P
LNF-I	-0.51 (1.75)	0.772	3.30 (2.66)	0.215
2'-FL	**-5.60 (1.93)**	**0.004**	3.77 (2.14)	0.078
LDFH-I	5.87 (2.95)	0.047	**-13.32 (5.33)**	**0.012**
LDFT	3.09 (4.74)	0.514	-16.82 (11.00)	0.126

[*]Each model included only one milk oligosaccharide (expressed in terms of percent of total oligosaccharide) as the independent variable and pathogen-specific diarrhea as the dependent variable. Negative beta coefficients indicate protection. Significant protective associations are in bold type.

[†]22 subjects experienced 31 cases of campylobacter diarrhea, 13 subjects experienced 16 cases of calicivirus diarrhea.

Figure legend

Figure 1. The incidence of *C. jejuni diarrhea,* calicivirus diarrhea and moderate to severe diarrhea of all causes in study children whose mother's milk contains low, medium, or high relative amounts of **(Panel A)** 2'-FL **(Panel B)** LDFH-I (Le^b), and (Panel C) total 2-linked fucosyloligosaccharide expressed as a percent of milk oligosaccharide. The bars indicate the pathogen-specific incidence rates in each group; the vertical lines indicate the standard error. The low, medium, and high groups each represent the oligosaccharide values of a tertile (n=31) of the study population. **Panel A**: For 2'-FL, the percentage of total oligosaccharide values are, by group: low (<0.29), medium (0.29–0.36), and high (>0.37). Overall protection against symptomatic *C. jejuni* infection was significantly (P=0.004) associated with increased 2'-FL (H-2) as a percentage of total milk oligosaccharides. Compared to the low tertile (2'-FL as a percentage of total milk oligosaccharide) group, campylobacter incidence in the medium and high tertile groups was significantly (P<0.01) reduced. **Panel B**: For LDFH-I, the percent of oligosaccharide values in each group are: low (<0.07), medium (0.07–0.11), and high (>0.12). Overall protection against symptomatic calicivirus infection was significantly (P=0.012) associated with increased LDFH-I (Le^b) as percentage of total milk oligosaccharides. Compared to the low tertile group, calicivirus incidence in the high tertile group was significantly (P=0.02) reduced. **Panel C**: For total 2-linked oligosaccharide, the percent of milk oligosaccharide values in each group are: low (<0.72), medium (0.72 – 0.77) and high (>0.77). Overall protection against moderate-to-severe diarrhea was significantly (P<0.001) associated with increased α 1,2-linked oligosaccharide. Compared to the low group, incidence in the medium and high groups was significantly (P<0.01) reduced.

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Abstract

Background: Laboratory data suggest that fucosylated oligosaccharides found in human milk protect against diarrhea cause by pathogens, but studies in infants are lacking. Methods: We examined human milk fucosylated oligosaccharide profile in relation to risk of diarrhea from all causes and from stable toxin-producing *E. coli* (STEC) infection in a cohort of 93 breast-fed Mexican infants from birth to the end of breast-feeding (median 9 months, range 0.7-24 months, 856 child-months of follow-up). Each week, field workers collected infant feeding and illness data and stool samples, which were analyzed for pathogens. A maternal milk sample collected 1-5 weeks postpartum was analyzed for 2-linked fucosylated oligosaccharides (fucose linked by α1,2 glycosidic bonds) and for 3/4-linked fucosylated oligosaccharides (fucose linked only by α1,3 or α1,4 glycosidic bonds). Results: STEC infections were detected in 47 infants. The mean quantity of milk oligosaccharides was unrelated to risk of diarrhea or STEC (STEC+ stool). However, infants whose milk had low ($\leq$5.9, n=23) vs. high ($>$5.9, n=70) ratios of 2-linked to 3/4-linked oligosaccharides had more episodes of moderate-to-severe diarrhea from any cause (14.3 vs. 22.3 cases/100 child-months, Rate Ratio [RR]=0.64, 95% CI 0.46, 0.90; P<.01). The mean ratio of 2- to 3/4-linked fucosylated oligosaccharides was lower (P <0.01) in milk consumed by infants who were ever STEC+ symptomatic (3.9 $\pm$ 0.7[SE], n=4) vs. STEC+ asymptomatic (7.6 $\pm$ 1.0, n=43) or STEC- (7.5 $\pm$ 1.0, n=46). Conclusion: The α2- to α3/4-linked fucosylated oligosaccharide ratio may be a marker for human milk molecules that protect against various causes of diarrhea. The study is the first to demonstrate that variation in human milk oligosaccharide profile is significantly associated with risk of diarrhea in breast-fed infants.

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Introduction

Breast-feeding provides significant protection against morbidity and mortality due to infant diarrhea (1-4). A pooled analysis of data from developing countries found that lack of breast-feeding was associated with five-fold increased risk of infant mortality in the first year of life (2). Human milk confers protection to infants against infectious diseases through a variety of bioactive substances including secretory antibodies, multifunctional agents, and oligosaccharides (5,6). While the varied bioactive factors in human milk offer significant protections against infectious diseases, the protection is not absolute. Some infants, even when breast-fed, experience multiple diarrhea episodes of moderate-to-severe diarrhea. While efforts are undertaken to protect, promote, and support breast-feeding worldwide, it is also important to understand human milk immunology and the variation in risk of disease that occurs among breast-fed infants.

Much of the risk of diarrhea in breast-fed infants can be explained by a degree of breast-feeding, i.e., partial versus exclusive breast-feeding (ref), and/or adverse environmental factors that result in high dose exposure to pathogens (ref). However, research on human milk also has demonstrated variation between mothers in the relative concentration of protective factors in their milk, e.g., secretory antibodies, glycoconjugates, and oligosaccharides (refs), which has been associated with variation in protection against specific infectious diseases (ref).

Human milk oligosaccharides constitute the third most abundant constituent of human milk, after lactose and fat, and form a critical part of the innate defense system. Yet studies have shown that the oligosaccharide profile of human milk varies significantly between mothers and

over the course of lactation (ref). For example, the ratio of α1,2 to α1,3 or α1,4-linked fucosyl oligosaccharides changing from approximately 5:1 at one week to 1:1 at one year postpartum.

In vitro and in vivo studies have shown that the fucosylated oligosaccharide fraction of human milk inhibits stable toxin (ST) associated *E. coli* and ST-induced secretory diarrhea. Evidence also exists of protection by fucosylated α1,2-linked oligosaccharides against other common enteric pathogens, including campylobacter and caliciviruses (ref). A recently completed preliminary study (unpublished) suggested that the ratio of α1,2-linked fucosylated oligosaccharides referent to the α1,3 and α1,4-linked oligosaccharides of maternal milk might be a biomarker of protection against symptomatic infection with ST-associated *E. coli*.

Between 1988 and 1991 a longitudinal study of human milk protection against enteric infection was undertaken in Mexico City. We analyzed a subset of breast-fed infants in that cohort to determine the fucosylated oligosaccharide pattern found in maternal milk and its relationship to the relative risk of infant diarrhea in general, and ST-associated diarrhea in particular.

Methods and materials

Study design and population

The study site was San Pedro Martir, a low-income neighborhood of Mexico City. The enrollment criteria and recruitment methods have been published previously (Velazquez, 1993). A cohort of 316 mother-infant pairs was enrolled beginning in March 1988 and monitored for up to two years. From this total cohort, the milk samples of 99 mother-infant pairs were selected to survey the oligosaccharide concentration in their milk samples. Infants were eligible to be

included for the study if they were breastfed from birth, had follow-up information beyond 2 weeks, and had an adequate volume of milk sample collected between weeks 1-5 postpartum.

This research was approved by the institutional review boards of the Instituto Nacional de la Nutricion in Mexico, and the University of Texas Health Science Center at Houston. Parents of all infants enrolled in this study were briefed on the purpose and procedures of the study and signed a written consent form.

Definition of Disease State

A diarrhea episode was defined as three or more watery stools within a 24 hour period, or loose-to-watery bowel movements that exceeded , by two stools, the child's usual stool frequency during the previous 4 weeks. The end of a diarrhea episode was defined as the first day during which bowel movements returned to their usual daily pattern. Severity of diarrhea was assessed by the numerical scoring system of Ruuska and Vesikari (13). An infection with stable toxin-associated E. coli (STEC) was defined as the detection of STEC in stool samples. An infection with STEC was classified as asymptomatic if a child had no symptoms of at the time of the stool collection. Similarly, an infection was defined as symptomatic if diarrhea occurred at the time of the stool sample collection.

Sample Collection

The entire milk content of one breast was collected by breast pump between 0800 hours and 1200 hours, transported on ice, and, within 4 hours of collection, frozen and stored at -70°C. Milk was collected weekly for 4 weeks post partum, then monthly until weaning. Stool samples were collected weekly from each child, and additional stool samples were obtained whenever

diarrhea occurred. Stool samples were kept on ice, transported within 3 hours of collection to the laboratory, and processed within 24 hours.

Analysis of Milk Oligosaccharides

Milk oligosaccharides were isolated as described previously (14 Chaturvedi *et al.*, 1997). Briefly, milk samples (0.9 ml) were thawed immediately before use. After addition of water (0.9 ml), the samples were centrifuged at 4000 x g for 45 min at 4°C. The solidified layer of fats and lipids was removed by pipetting from the lower, aqueous layer. The proteins and a portion of lactose were precipitated overnight at 4°C after the addition of ethanol to a final concentration of 66.7%. The precipitate was removed by centrifugation at 4000 x g for 15 min at 4°C. The clear supernatant was then dried under nitrogen and lyophilized.

Sodium borohydride ($NaBH_4$) and dimethylaminopyridine (DMAP) were purchased from Aldrich Chemical Company (Milwaukee, WI). Benzoic acid was obtained from Sigma Chemical Company (ST. Louis, MO), and the AG50WX8 (H^+) and AG1-X8 (OAc^-) ion exchange resins (100-200 mesh) were obtained from Bio-Rad (Hercules, CA). Before use the AG50 resin was converted to the pyridinium form by treatment with aqueous pyridine. HPLC grade acetonitrile was purchased from Fisher Scientrific (Pittsburgh, PA). Double-distilled de-ionized water was used from all HPLC analyses. All the other chemicals and solvents were analytical grade. Human milk oligosaccharide standards were purchased from Oxford GlycoSystems (Rosedale, NY).

Statistical Analysis

The number of age-specific child-months at risk for diarrhea was calculated for each child. The number of child-months at risk for a first diarrhea episode was calculated from birth to the date of onset of the first episode of diarrhea. The number of child-months at risk for a subsequent episode was defined by the interval between episodes. The time at risk for a child who dropped out of the study was included for the respective period of observation. The outcomes assessed were all diarrhea episodes, mild or moderate-to-severe diarrhea episodes, and symptomatic and asymptomatic ST infections. The chi-square test or t-test was used to compare groups as appropriate.

A graphic display of the data was used to examine pair-wise relations between α1,2 and α1,3/4 linked oligosaccharides. When scatter plots suggested linear relations, the Pearson correlation coefficient was estimated to examine significance.

Results

Study Population

The 93 mother-infant pairs included in this analysis were monitored for 856 child-months. Mothers were on average 24 years of age, 55% had completed a secondary or higher education. Half of the infants were female, and 64% were either the first or second born child. Forty-two percent of mother-infant pairs lived in households with 6 or more people. The mean duration of breastfeeding was 9 months. The median number of diarrhea episodes was 2 per child over the course of the breastfeeding follow-up time, with a median of one moderate-to-severe diarrhea episode per child.

Full cohort, [N=289, but with same criteria as sub-study, N=273]. No significant differences were seen between those in this analysis and those who were not included with respect to the mother's age, the gender of the infant, the birthing order of the infant, or the number of people living within the homestead. However, mothers included in the analyses were more likely to complete a secondary education or higher (RR=1.69, p=0.004). The mean duration of breastfeeding for the 93 subjects included in the oligosaccharide analyses was significantly longer than the 180 subjects not included (mean duration 9 months vs. 7 months, p=0.006).

Oligosaccharide Profile

As seen in figure 1, α1,2-linked were (slightly?) correlated with α1,3/4-linked oligosaccharides (r=0.47, p<0.001). The distribution of oligosaccharide concentrations found in maternal milk ranged from 56 to 1489 for α1,2-linked (median 637) and from 8 to 864 for α1,3/4-linked (median 115). The ratio between these two structures was (describe range, ???) (median 5.91). The groups of infants with high (>5.91) and low (≤5.91) α1,2 to α1,3/4 oligosaccharide ratios had a similar profile in terms of the demographic characteristics described above and shown in Table 1.

Incidence of Diarrhea

A total of 238 diarrhea episodes were identified during the 856 months of follow up. Of these 238 cases, 150 were identified as moderate-to-severe cases. The overall incidence rate of diarrhea for the 93 subjects from birth to the end of breastfeeding or 2 years was 27.8 per 100 child-months. The incidence rate of moderate-to-severe diarrhea was 18.5 per 100 child-months.

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As seen in Table 1, those with a high oligosaccharide ratio (>5.91) had lower rates of both overall diarrhea incidence (24.8 vs. 33.5 per 100 child-months; RR=0.74, p=0.21) and moderate-to-severe diarrhea incidence (14.3 vs. 22.3 per 100 child-months; RR=0.64, p=0.009) compared to those with a low oligosaccharide ratio (≤5.91). No age trend was observed. (elaborate??)

Describe overall pattern, # cases

Table 3

Indicate no clear evidence of dose response when data analyzed by cut point of protection not clear

ST E. coli-Associated Diarrhea

Stable toxin producing *E. coli* was identified in only 4 children. No repeated symptomatic ST infection occurred in any of the 4 infected children. Of the 93 subjects, 43 (46%) had an asymptomatic infection and the remaining 46 (49%) were never infected.

Risk of *E. coli* Associated Diarrhea

4 cases, moderate-to-severe, describe figure 3.

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Table 1. Description of the study population characteristics

Characteristic		**Total**	**High Ratio**	**Low Ratio**
		No. (%)	**N=47**	**N=46**
Age of mother:	<20	22 (24%)	12 (26%)	10 (22%)
	≥20	71 (76%)	35 (74%)	36 (78%)
Infant gender:	Male	43 (46%)	23 (49%)	20 (43%)
	Female	50 (54%)	24 (51%)	26 (57%)
Maternal Education:				
	None/Elementary	42 (45%)	21 (45%)	21 (46%)
	Secondary	31 (33%)	16 (34%)	15 (32%)
	Beyond Secondary	20 (22%)	10 (21%)	10 (22%)
Number of people in household:				
	3	16 (17%)	8 (17%)	8 (17%)
	4	19 (20%)	9 (19%)	10 (22%)
	5	19 (20%)	8 (17%)	11 (24%)
	≥6	33 (42%)	22 (47%)	17 (37%)
Birth order of infant:				
	1st	31 (33%)	18 (38%)	13 (28%)
	2nd	31 (31%)	14 (30%)	17 (37%)
	≥3rd	31 (31%)	15 (32%)	16 (35%)

Table 1 cont'd.

Duration of breastfeeding, months:		**Total**	**High Ratio**	**Low Ratio**
	<3	23 (25%)	14 (30%)	9 (20%)
	3-5	10 (11%)	6 (13%)	4 (8%)
	6-8	13 (14%)	7 (15%)	6 (13%)
	9-11	15 (16%)	6 (13%)	9 (20%)
	≥12	32 (34%)	14 (29%)	18 (39%)
Living in Brick House		90 (97%)	47 (100%)	43 (93%)
Hygiene (animals present)		66 (71%)	29 (62%)	37 (80%)
Diarrhea episodes/child,	no.			
	0	25 (27%)	16 (34%)	9 (20%)
	1	12 (13%)	5 (11%)	7 (15%)
	2	21 (22%)	10 (21%)	11 (24%)
	3	8 (9%)	6 (13%)	2 (4%)
	≥ 4	27 (29%)	10 (21%)	17 (37%)
Moderate-to-severe episodes/child,				
	no.			
	0	45 (48%)	26 (55%)	19 (41%)
	1	12 (13%)	6 (13%)	6 (13%)
	2	12 (13%)	6 (13%)	6 (13%)
	3	9 (10%)	4 (8%)	5 (11%)
	≥ 4	15 (16%)	5 (11%)	10 (22%)

Table 2. Incidence of diarrhea cases per 100 child-months of breastfeeding among infants consuming maternal milk containing high vs. low ratios of α1,2-linked to α1,3/4-linked fucosylated oligosaccharide quantities

		Oligosaccharide			
Outcome measure		High N=47*	Low N=46*	RR (95%CI)	p-value
All diarrhea	Cases, no.	95	143		
	Incidence	24.8	33.5	0.74(0.57,0.96)	0.021
Mild diarrhea	Cases, no.	40	48		
	Incidence	10.4	11.3	0.93(0.61,1.41)	0.720
Moderate-to-severe diarrhea	Cases, no.	55	95		
	Incidence	14.3	22.3	0.64(0.46,0.90)	0.009

Table 3. Number of episodes of diarrhea

		All diarrhea		Moderate-to-severe	
Episode No.		High	Low	High	Low
1st episode	Cases, no.	31	37	21	27
	Incidence	20.6	20.3	9.31	11.5
2nd episode	Cases, no.	26	30	15	21
	Incidence	10.1	11.5	4.8	6.8
3rd episode	Cases, no.	16	19	9	15
	Incidence	4.9	6.1	2.5	4.3
4th episode	Cases, no.	10	17	5	10
	Incidence	2.8	4.8	1.3	2.6
5th episode	Cases, no.	7	13	2	7
	Incidence	1.9	3.4	0.5	1.7

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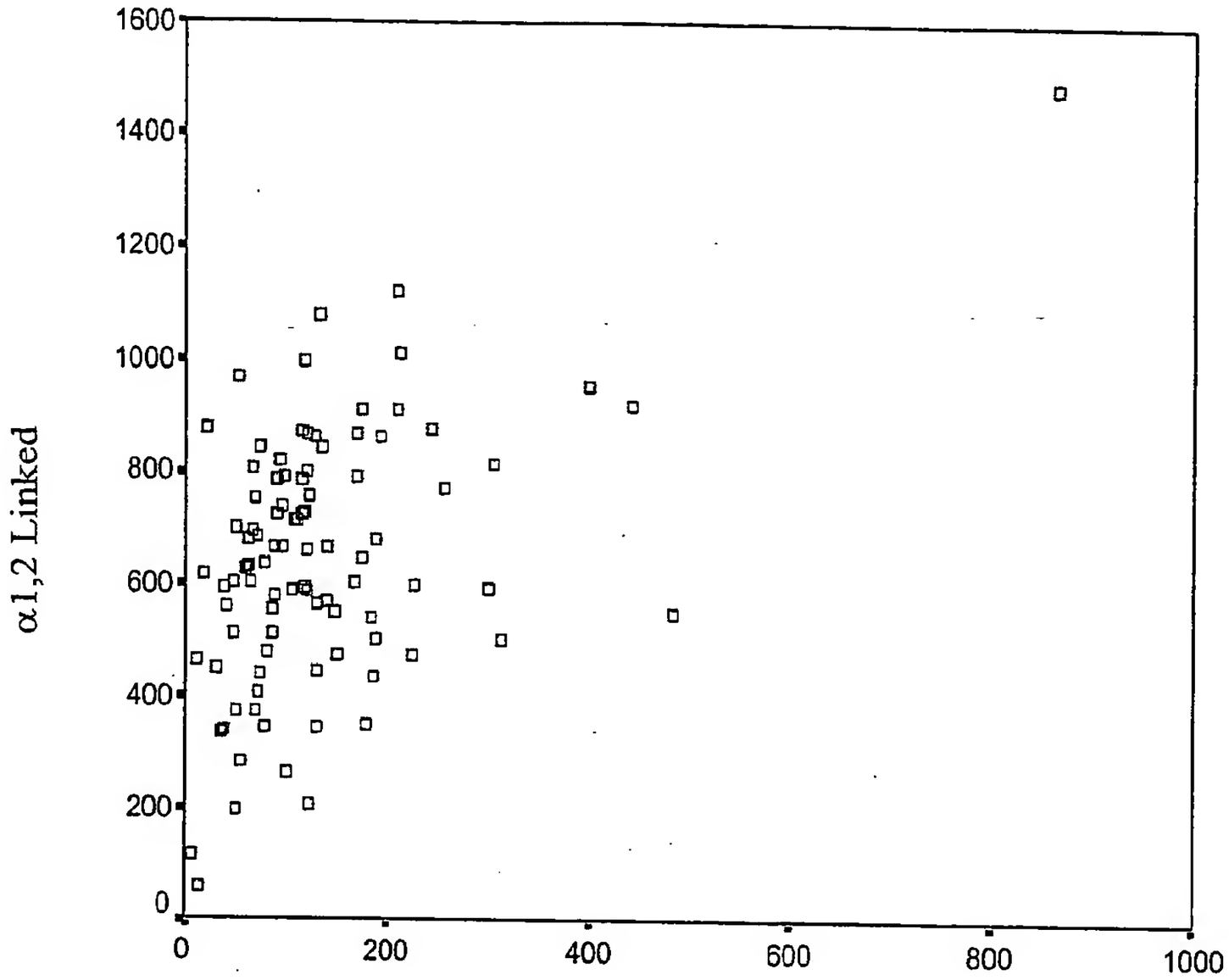
Figure 1. Scatterplot of α1,2 Linked and α3/4 Linked oligosaccharides*



*Pearson correlation with outlier, r=0.472, p<0.001. Removal of outlier, r=0.316, p<0.01.

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Figure 2.

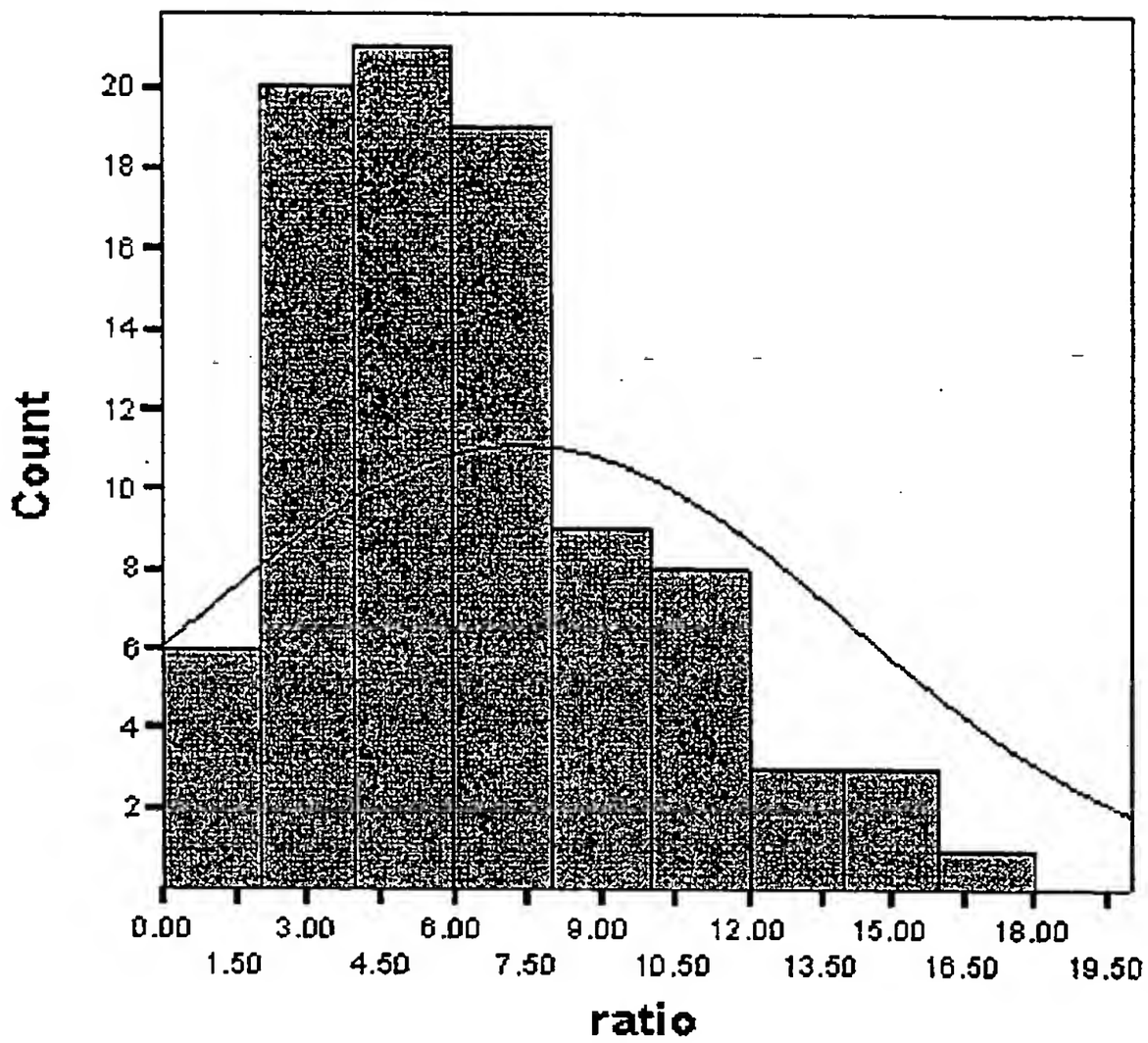

Figure 3. Mean ± standard error (bar and line) by stable toxin-producing *E. coli* infection status

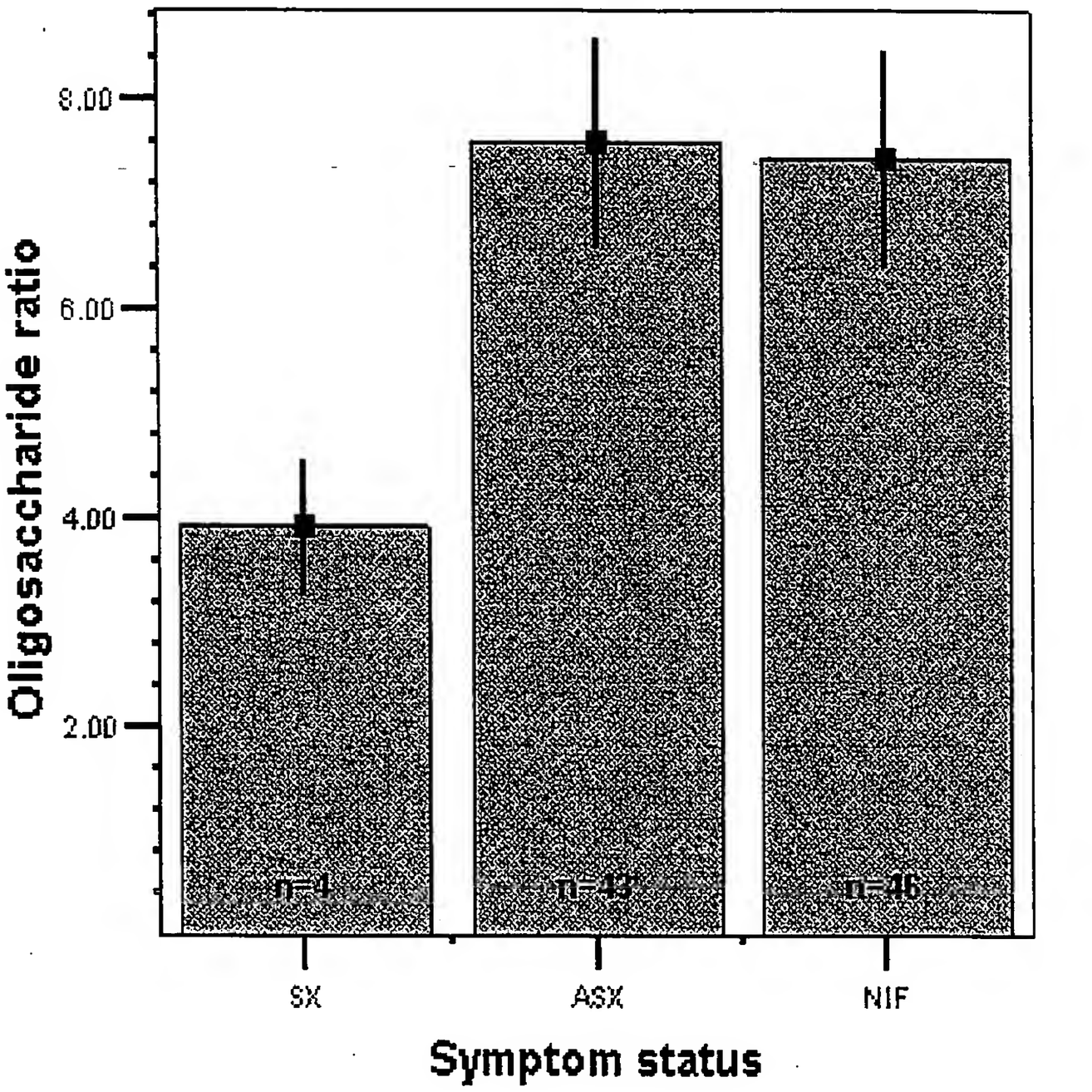

Figure 1. Frequency distributions of 2-linked oligosaccharides (Panel A), 3 / 4-linked oligosaccharides (B), and the ratio of these components (C) in individual maternal milk samples

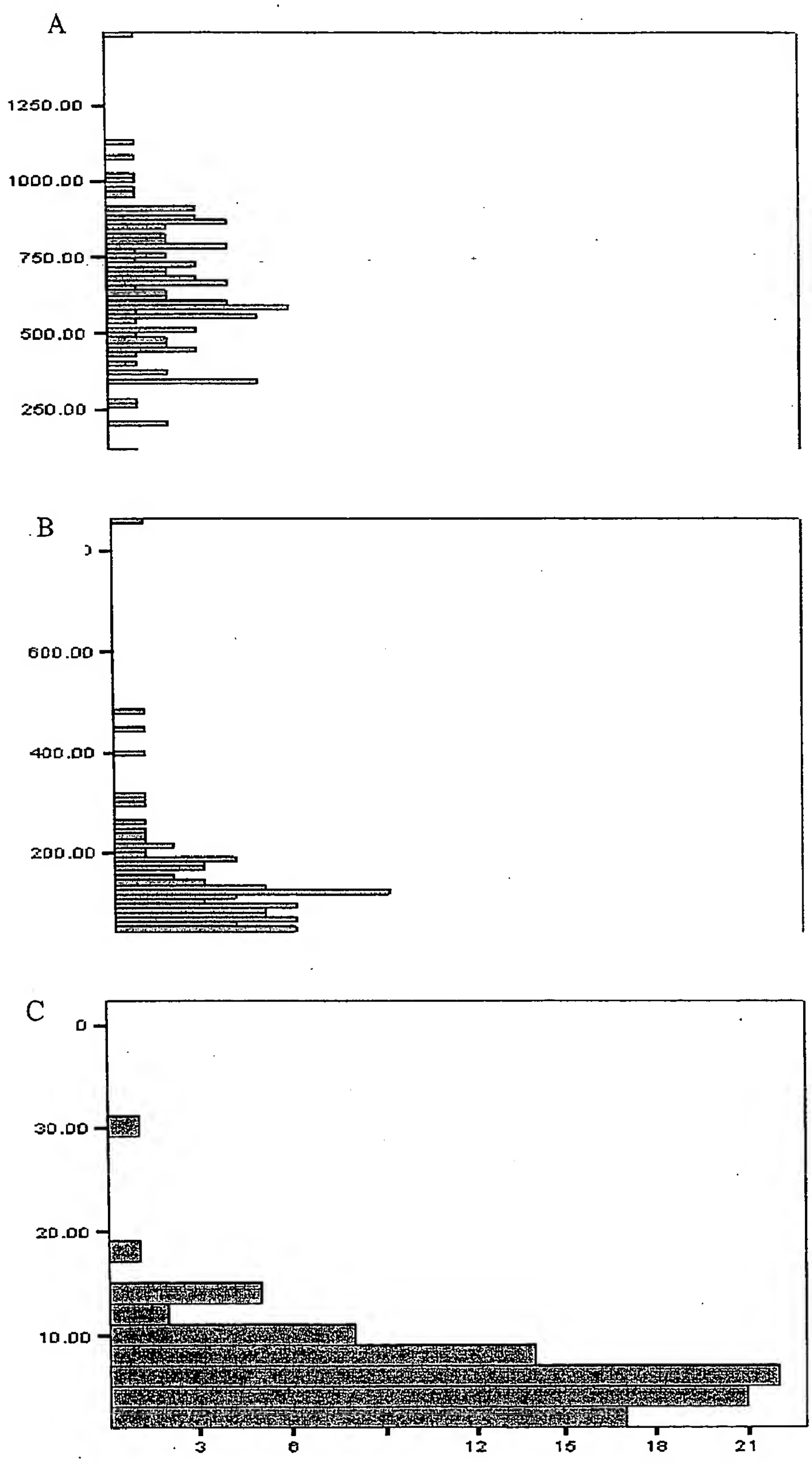

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Figure 2. Mean ± standard error (bar and line) by stable toxin-producing *E. coli* infection status

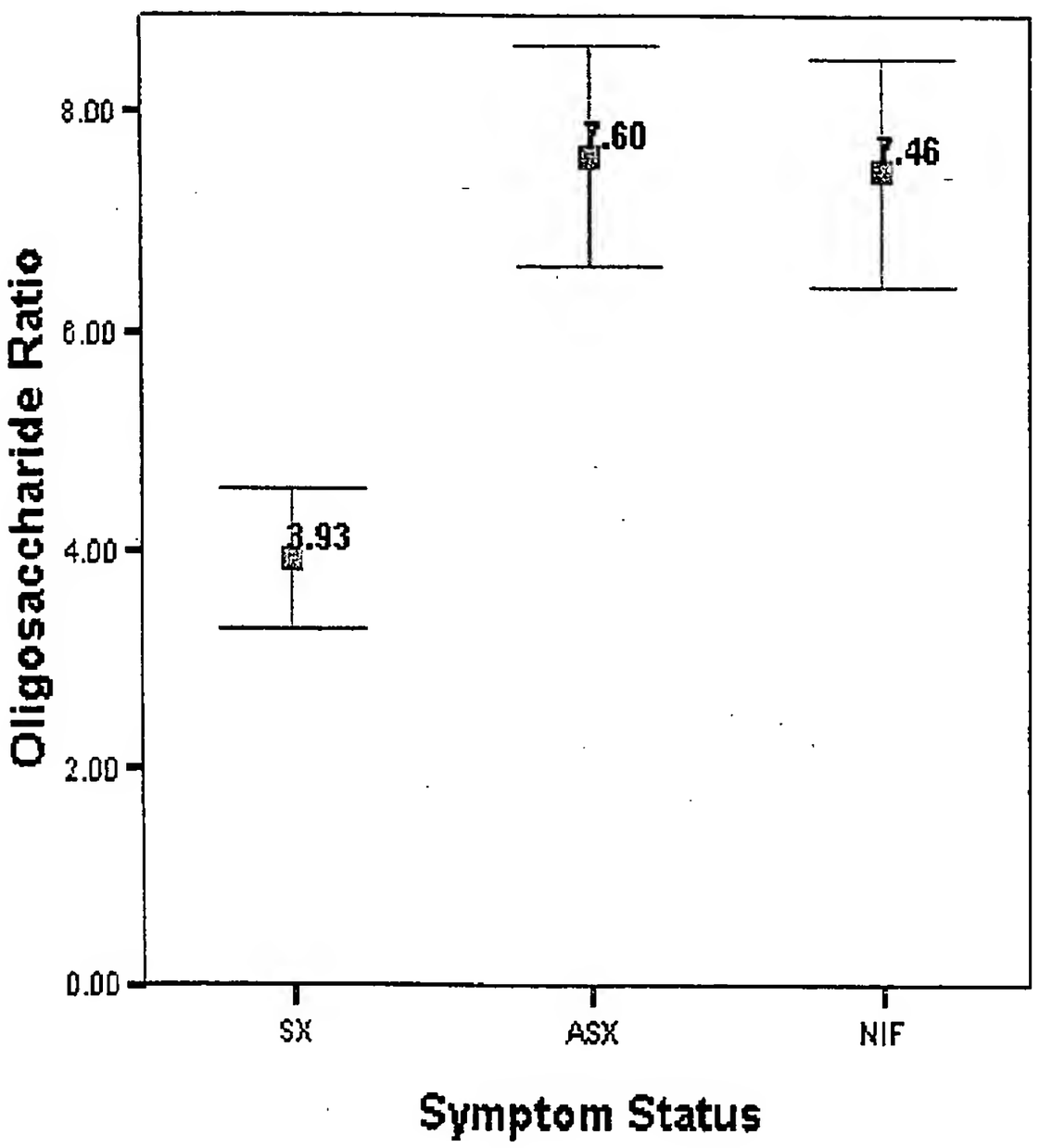

Table 2. Distribution of subjects, oligosaccharide concentration, and incidence of diarrhea by the oligosaccharide ratio and Lewis blood group categories

Oligosaccharide Ratio		Lewis Blood Group Type a-b+ n=67	a-b- n=24	a+b- n=2
Low (n=23)	No. subjects	23	0	0
	2-linked concentration	610.0		
	3/ 4-linked concentration	259.5		
	Incidence of diarrhea	36.2		
Medium (n=47)	No. subjects	38	7	2
	2-linked concentration	653.5	721.0	647.0
	3/ 4-linked concentration	109.5	137.8	116.5
	Incidence of diarrhea	27.4	29.9	5.4
High (n=23)	No. subjects	6	17	0
	2-linked concentration	750.2	601.5	
	3/ 4-linked concentration	64.5	48.1	
	Incidence of diarrhea	26.0	26.8	

What is claimed is:

1. A method for evaluating phenotype selected from the group consisting of:
   a) solid phase enzymatic assay for rapid screening of secretor status in saliva or milk;
   b) HPLC to measure oligosaccharides of human milk and other biological samples;
   c) capillary electrophoresis of acidic oligosaccharides of milk or other tissues; and
   d) glycochip for screening specific pathogen binding to carbohydrate receptors and other determinants.

2. A method for evaluating genotype comprising:
   a) RFLP-PCR to determine genotypes relevant to susceptibility, snapshot, PCR, and real time PCR.

3. A method for synthesizing fucosylated glycans through chemi-enzymatic modification of yeast glycans.

4. A method for synthesizing fucosylated glycans through genetic modification of yeats to induce the production of fucosylated glycans.

5. Anti-infectious agents containing fucosylated monovalent epitopes.

6. Anti-infectious agents containing fucosylated multivalent epitopes.

7. Anti-infectious agents containing fucosylated polyvalent epitopes.

8. Anti-infectious agents containing mixtures of monovalent glycans.

9. Prophylactic agents containing fucosylated monovalent epitopes.

10. Prophylactic agents containing fucosylated multivalent epitopes.

11. Prophylactic agents containing fucosylated polyvalent epitopes.

12. Prophylactic agents containing mixtures of monovalent glycans.

13. Therapeutic agents containing fucosylated monovalent epitopes.

14. Therapeutic agents containing fucosylated multivalent epitopes.

15. Therapeutic agents containing fucosylated polyvalent epitopes.

16. Therapeutic agents containing mixtures of monovalent glycans.